

## **Historic, Archive Document**

Do not assume content reflects current scientific knowledge, policies, or practices.



A61.9  
Se 3  
Cop 2

UNITED STATES DEPARTMENT OF AGRICULTURE  
AGRICULTURAL RESEARCH SERVICE

# Proceedings

## SEED PROTEIN CONFERENCE

January 21-23, 1963

U.S. DEPT. OF AGRICULTURE  
AGRICULTURAL LIBRARY  
OCT 28 1964  
CURRENT SERIAL RECORDS

SOUTHERN UTILIZATION RESEARCH AND DEVELOPMENT DIVISION  
1100 Robert E. Lee Boulevard  
New Orleans 19, Louisiana





# C O N T E N T S

	Page No.
Foreword	iv
Summary of Conference	1
K. F. Talluto and A. M. Altschul	
Welcome Remarks	11
C. H. Fisher	
Opening Remarks	13
G. W. Irving, Jr.	
Seed Proteins - A Perspective	17
A. M. Altschul	

## FIRST SESSION

G. R. Tristram, Chairman

Principal Properties of Wheat Albumins	21
C. C. Nimmo	
Structural Investigations of Wheat Gliadin and Glutenin	27
J. H. Woychik	
Immunochemical Studies on Wheat and Barley Proteins	40
P. Grabar, N. Benhamou and J. Daussant	

## SECOND SESSION

M. L. Anson, Chairman

Recent Advances in the Chemistry of Soybean Proteins	43
W. J. Wolf	
Some Properties of Purified Edestin	56
D. M. Stockwell, J. M. Dechary and A. M. Altschul	
Soluble Proteins of the Peanut Cotyledon; Purification and Properties of $\alpha$ -Conarachin	66
J. M. Dechary	

### THIRD SESSION

N. Sharon, Chairman

Seed Hemagglutinins I. E. Liener	69
Isolation and Characterization of Soybean Trypsin Inhibitors J. J. Rackis	98

### FOURTH SESSION

J. C. Perrone, Chairman

Oilseed Allergens J. R. Spies	109
Phytochrome--The Conjugated Protein Controlling the Photoresponsive Growth of Plants H. W. Siegelman	113

### FIFTH SESSION

V. L. Frampton, Chairman

Protein Bodies, Nucleic Acids, and Enzymes of Peanut Cotyledons: Changes on Germination J. H. Cherry	115
Enzyme Formation in the Cotyledons and Endosperm of Germinating Seeds J. E. Varner and G. Schidlovsky	145
Lysosome-Like Behavior of Acid Phosphatase in Germinating Onion Seeds J. F. Harrington and A. M. Altschul	191

### SIXTH SESSION

M. L. Rollins, Chairman

Protein in the Developing Soybean Cotyledon R. W. Howell	193
Intracellular Location of Zein in Maize D. N. Duvick	196

## SEVENTH SESSION

H. L. Wilcke, Chairman

Fractionation of Particulate Proteins of the Peanut Cotyledon	218
A. M. Altschul, W. J. Evans, A. A. Woodham, W. B. Carney, and J. M. Dechary	
The Proteins of Cottonseed Pigment Glands	229
W. H. Martinez and L. C. Berardi	
Lipid-Protein Particles in the Cottonseed	240
L. Y. Yatsu	

## EIGHTH SESSION

M. A. Stahmann, Chairman

Nonenzymatic Cleavage of Peptide Bonds	244
E. Gross	
Cyanate as a Reagent in Protein Chemistry	248
G. R. Stark	
Interaction of Brom Thymol Blue with Proteins and its Relation to their Conformation	250
E. Antonini	

## NINTH SESSION

A. A. Altschul, Chairman


Optical Rotation in Relation to Protein Structure	252
B. Jirgensons	
Analytical and Preparative Electrophoresis on Polyacrylamide Gel	275
W. J. Evans	
Luncheon Program	
Introduction of Speakers Following Luncheon	280
M. Milner	
Luncheon Talk - The Future of Isolated Oilseed Protein in Foods	281
M. L. Anson	
Attendance List	285

## FOREWORD

In recent years there has been increasing attention given to plant proteins. They are the greatest source of protein food for man and animal and offer opportunities for studies in protein synthesis and secretion and in protein structure.

This conference was sponsored by the Southern Utilization Research and Development Division to bring together leaders in this field of research to review the progress that has been made on this group of proteins and on the role they play in seeds, and to try to project the direction of research on this class of compounds.

This Proceedings presents in summary or in full the discussions of the participants.

  
C. H. Fisher, Director

Southern Utilization Research & Development Division  
Agricultural Research Service  
U. S. Department of Agriculture

The statements contained in the speeches reproduced in this proceedings of the Conference are those of the speakers and do not necessarily indicate or reflect the view of the U.S.D.A.

No information in this report should be reproduced or used in any way without specific permission from the author or organization.



## SUMMARY OF CONFERENCE

Katherine F. Talluto and Aaron M. Altschul  
Southern Utilization Research and Development Division  
New Orleans, Louisiana

Perhaps for the first time in modern history a conference was devoted exclusively to seed proteins. This gathering was made possible by the resurgence of interest in seed proteins, in their isolation and chemistry, and in their role in plant physiology. Aaron M. Altschul (USDA, New Orleans), Chairman of the Conference, pointed out in his introductory remarks that although seed proteins always have been of practical interest as major sources of protein food for man and animal, there is a growing realization that seeds and seed proteins can serve as biological models for study of problems in growth and differentiation and for clarification of certain aspects of protein chemistry.

The program included the following subjects:

1. Properties of pure seed proteins.
2. Questions on organization and conformation of seed proteins.
3. Recent progress in analysis and purification of proteins.
4. Isolation and properties of certain biologically active seed proteins.
5. Subcellular location of seed proteins.
6. Synthesis of enzymes during germination.

Despite their low solubility in solutions of low ionic strength and moderate pH and their strong tendency for interaction, progress has been reported in analyzing for and purifying seed proteins. J. M. Dechary (USDA, New Orleans) reported on the isolation of  $\alpha$ -conarachin by chromatography of peanut proteins on DEAE cellulose. It is monodisperse by

criteria of ultracentrifugation and chromatography, but shows a second minor component upon electrophoresis on polyacrylamide gel. W. J. Evans (USDA, New Orleans) described analytical electrophoresis on polyacrylamide gel and showed how this medium could be used in preparative electrophoresis. Such a procedure is particularly advantageous for separating highly interacting proteins as occur in mixtures of seed proteins. Other examples of the usefulness of polyacrylamide gel electrophoresis in the characterization of various proteins were furnished: wheat albumins by C. C. Nimmo (USDA, Albany, Calif.) and cottonseed pigment gland proteins by W. H. Martinez (USDA, New Orleans). Martinez found that these proteins differ in amino acid content from the major cytoplasmic proteins of the cottonseed. One of the fractions contained a significant amount of glucosamine.

J. H. Woychik (USDA, Peoria, Illinois) described studies on the wheat proteins gliadin and glutenin that were undertaken to compare the type of disulfide bonding in these components and to determine whether glutenin (M.W. 2-3 million) consists of the components of gliadin (M.W. about 40,000) joined randomly through intermolecular disulfide bonding. Starch gel electrophoresis after reduction and alkylation showed an increase in the number of components of glutenin, indicating extensive intermolecular bonding; but there was no change in the number of gliadin components, indicating predominantly intramolecular bonding. Essentially all of the components in reduced glutenin correspond in mobility to reduced gliadin, although major differences in distribution of components were apparent. One of the gliadin components,  $\gamma$ -gliadin, was isolated and

found to have a high content of proline and glutamine (20 and 40% by weight, respectively). The failure to observe a decrease in molecular weight after reduction, a single component on starch gel electrophoresis after reduction, and the finding of one N-terminal residue suggest a single-chain structure.

D. M. Stockwell (USDA, New Orleans) solved the problem of low solubility of hempseed proteins by chromatographing at 50° C. on DEAE cellulose. By such means it was possible to show that edestin, a crystalline protein from hempseed, was not pure by chromatographic standards. He was able to isolate a fraction of higher purity and greater solubility.

Purified seed proteins have been shown to have molecular weights from below 20 thousand in urea to over a million in certain associated states. This phenomenon was true for the proteins of the soybean as reported by W. J. Wolf (USDA, Peoria) and for the proteins of the peanut and hempseed. Some ideas on the conformation of plant proteins were discussed by B. Jirgensons (M. D. Anderson Hospital and Tumor Institute, Houston), who compared optical properties of  $\alpha$ -conarachin from peanuts, an edestin fraction, and some trypsin inhibitors from soybeans and peas with other known proteins. Alpha-conarachin and edestin are in the group of nonhelical proteins. Trypsin inhibitor from soybeans was also in this group, but the trypsin inhibitor from peas had a high helical content. The Moffitt parameter,  $b_0$ , is near zero for  $\alpha$ -conarachin and edestin, and shifts toward negative values upon denaturation with detergents. However, denaturation in 8 M urea had no effect on the  $b_0$  value of edestin; but the levorotation was strongly shifted to the negative.



When plant proteins are classified according to their rotatory dispersion properties, examples can be found for every group: highly helical, low helix content, and nonhelical. But when the sample is restricted to major seed globulins -- legumin from peas, arachin and  $\alpha$ -conarachin, edestin, and amandin from almonds -- the optical activity is of about the same magnitude and their specific rotation is shifted in a negative direction upon denaturation. We seem to be dealing here with a group of proteins of little or no  $\alpha$ -helical conformation. Whatever order they have may be contributed by hydrophobic bonding.

E. Antonini (Universita di Roma) reviewed the technique of studying the effect of affinity of a dye for a protein on its conformation and described examples from applications of this technique to hemoglobin and myoglobin and their derivatives. He commented that the behavior of seed proteins was similar in many respects in terms of association-dissociation properties, solubility, and lack of helical content to apo-enzymes.

The complexity of seed proteins was emphasized by immunoelectrophoretic studies reported by Pierre Grabar (Institut Pasteur) on the proteins of wheat, barley, and rye. There is no relationship between fractionation by starch gel electrophoresis and number of immunochemically different components, nor was there any meaning to the classical separation of the proteins of wheat into globulins and albumins on the basis of solubility; both groups contained proteins with the same antigenic properties. The wheat proteins were solubilized in acid and urea, or by polymerization of di-alanine chains onto the major protein species. Such modified proteins showed better solubility but the same immunological specificity. Total extracts of barley showed more protein components than did wheat. Some of



these, however, were found to be analogous or even identical, suggesting a common origin for these two grains. (Hybrids of wheat and barley gave all the proteins of both by immunoelectrophoresis.) Proteolytic enzymes were found in both wheat and barley.

M. L. Anson (London) cautioned that further interpretation of peaks obtained by chromatography is necessary and used the term "peakology" to describe the current tendency to rely too heavily on the results of a chromatogram. It must be ascertained that the presence of two peaks represents two proteins. For the most part, these peaks are not artifacts, but a variety of methods should be used to identify a protein, especially when the protein has no enzymatic activity.

George R. Stark (Rockefeller Institute) discussed cyanate as a reagent in protein chemistry. Cyanate reacts with charged rather than uncharged amino groups, a property enabling the comparison of the reactivities of  $\epsilon$ -NH<sub>2</sub> groups in proteins at neutral pH values, rather than at the more alkaline values required for reaction with most other reagents. The carbamylated derivatives may be cyclized to give hydantoins which can be separated from the mixture and hydrolyzed to give back amino acids. In this manner N-terminal amino acids and, as suggested by Nathan Sharon (Weizmann Institute, Israel),  $\epsilon$ -lysine values may be determined, and hence, lysine availability. Methods for determination of the availability of other amino acids might develop from studies such as described by E. Gross (N.I.H., Bethesda). He reviewed the possibilities for nonenzymatic splitting of peptide bonds. Of particular interest was the cyanogen bromide splitting of bonds involving methionine.

There were several reports on isolation and characterization of biologically-active proteins in seeds. I. E. Liener (U. of Minnesota) isolated the soybean hemagglutinin containing 10% by weight of glucosamine. He also observed that the hemagglutinin activity could be inhibited by a variety of sialic acid-containing mucoproteins. J. R. Spies (USDA, Washington) isolated several oilseed allergens, one of which contained 40% arginine. J. J. Rackis (USDA, Peoria) isolated soybean trypsin inhibitors by DEAE cellulose chromatography. One of these was identical with the classical soybean trypsin inhibitor first isolated by Kunitz. H. W. Siegelman (USDA, Beltsville, Md.) described the isolation and concentration of the very interesting light-sensitive phytochrome from germinating corn seedlings.

The evidence from cytology and particle fractionation was overwhelming that the major proteins of all seeds examined exist in subcellular particles. J. E. Varner (R.I.A.S., Baltimore) provided electron microscopy evidence for the existence of protein bodies in pea seeds and was able to isolate the major globulin by careful particulate separation. He showed that mitochondria are fully formed in the quiescent pea and can be observed after addition of water.

J. H. Cherry (Purdue) presented photomicrographs which showed the degradation of storage protein in the ectyledonary cells of the peanut (Arachis hypogaen). As the seeds germinate, the protein bodies swell and develop cavities. Later these swollen bodies are broken up, and the many fragments are digested and disappear. In a given cell population there is a wide range of protein body degradation, the degree of degradation being

related to the distance from the nearest vascular bundle. The cytoplasm appears as a three-dimensional network, which is between and connected to the subcellular particles in the resting seed. This network fragments and is no longer visibly intact after two days germination. Cherry also reported that the RNA content of the peanut seed upon germination increases three-fold by the eighth day, followed by a rapid decline thereafter. The DNA content doubled by the tenth day, then declined.

R. W. Howell (U.S.D.A., Urbana, Illinois) reported that in immature soybean seeds only about half the nitrogen is as protein, but the proportion of protein increases steadily with age and usually includes about 95% of the total nitrogen at maturity. At that stage, osmiophilic bodies (presumably protein bodies) are clearly visible throughout the parenchyma cells. The proportion of nucleic acids is relatively constant on a weight basis, but the soluble nucleotides show a decrease during seed development comparable to that of the total nonprotein nitrogen, which decreases as the protein content increases. Howell postulated that the protein in seeds probably serves a dual function: first, as enzymes and other metabolic components; and secondly, as metabolically inactive storage protein. D. N. Duvick (Pioneer Hi-Bred Corn Company, Johnston, Iowa) demonstrated two subcellular locations for proteins in corn: zein was found in subcellular particles and glutelin in the cytoplasmic matrix.

Lipid-protein particles in cottonseed were described by L. Y. Yatsu (National Cottonseed Products Assoc., New Orleans). Cottonseed kernels contain large intracellular bodies similar to those observed in the peanut and other seeds; and within these bodies are areas which have a high affinity for osmium. These areas have been identified tentatively as



sites of location of lipid. Between the bodies within the cell is a network which also contains small bodies having highly osmiophilic properties. By macerating cells in the presence of tannic acid, Yatsu was able to locate two particles, one with 25% lipid and the other with 50% lipid. Both contained about 50% protein.

A. M. Altschul described techniques for the isolation of particulate proteins from the peanut cotyledon, providing biochemical evidence for the existence of the protein in particles within the seed. He described first a method involving a nonaqueous medium developed by Dieckert and Snowden based on a technique of Behrens. By macerating peanuts in a solvent consisting of oil and carbon tetrachloride, it was possible to isolate particles containing 11% and 13% nitrogen. The use of Carbowax 20 M in aqueous medium and of 0.25 M sucrose also enabled the isolation of some of the protein in particles. DEAE cellulose chromatography showed a similarity in elution patterns between the particles obtained in aqueous media and a fraction obtained by ammonium sulfate fractionation of the soluble proteins of the intact peanut. Aside from providing evidence for the particulate nature of the major proteins, this similarity suggests that the study of the seed protein is not confounded by interaction of the major groups during maceration of the entire tissue.

It seems that the storage tissues of seeds contain "packages" of reserve or storage materials. Aside from the well-known starch grains, it now appears that most of the protein, phytic acid, and lipid is in particulates. It may be that the major seed globulins are indeed reserve proteins, but their presence together with other storage materials in

subcellular particles suggests also a possible enzymic role in the synthesis of the other reserves, and a role as structural proteins providing the matrix for these particles.

In several instances it was shown that hydrolytic enzymes are synthesized in the hypocotyledonary axis of germinating seeds. Varner found that the synthesis of  $\alpha$ -amylase, which occurs in the aleurone grains of peas, is triggered by gibberelic acid. The inhibition of the formation of  $\alpha$ -amylase by dinitrophenol, anaerobiosis, p-fluorophenylalanine and chloramphenicol indicates de novo synthesis of the  $\alpha$ -amylase rather than activation of pre-existing proteins. J. F. Harrington (USDA, New Orleans) described lysosome-like behavior in the development of acid phosphatase in germinating onion seeds. All of the activity was in the growing portion, and 80% was bound and could be released by incubation with Triton 100.

An informal program, "Some Comments on Food Applications of Seed Protein Concentrates," was presented at a luncheon meeting. Max Milner (UNICEF) Chairman, spoke of difficulties encountered by the United Nations in its efforts to bring into use vegetable proteins as foods because of lack of technology and of fundamental knowledge. M. L. Anson (Consultant, London) predicted that millions of pounds of protein isolates eventually will be made, and in different forms, for less than half the cost of these materials today. These products will resemble animal products; will be cheaper but not inferior; and may have keeping qualities, flavor, fat content, etc. superior to animal proteins. Industrial organizations should be aware that the production of protein isolates is a whole new

industry and should organize along lines best suited for the production of these materials. Z. I. Kertesz (Food and Agriculture Organization, Rome) outlined the qualities which new foods must possess to make them acceptable; the foods must be capable of local production, reasonably priced, easily transportable, nontoxic, eye-appealing, and not being utilized already as foods. Only one new food out of twenty becomes acceptable. Kertesz also emphasized the necessity of industry cooperation in the development of these new foods. He stressed that these foods will serve to supplement the existing diets, not replace them.

The level of understanding of seed proteins -- their chemistry and biochemistry -- displayed at this meeting was impressive. Many were led to feel that the continued study of these proteins on such a level would provide information of general value to protein chemistry and biochemistry. This understanding was also a portent of the future and gave rise to optimism in many quarters that the degree of sophistication required to make full use of seed proteins in foods could be achieved by the continuation of the basic developments reported at the meeting.

In a message to the meeting, George W. Irving, Jr., Deputy Administrator of the Agricultural Research Service, Washington, called this a unique and historic conference since it was probably the first one in modern history devoted solely to the subject of seed proteins. C. H. Fisher, Director of the Division, welcomed the scientists to New Orleans and E. L. Patton, Assistant Director, acted as Cochairman in charge of arrangements.



## WELCOME REMARKS

C. H. Fisher, Director  
Southern Utilization Research and Development Division  
New Orleans, Louisiana

Members of the Conference: It is my honor and pleasure, speaking for the Southern Division and myself, to welcome you to this Conference.

This is a special conference concerned with a most important topic and attended by distinguished experts from many parts of the world.

The international flavor of this conference has been present since the early planning stages. We are pleased because so many have come so far to enrich this conference with their participation. It is my understanding that about nine foreign countries are represented here by about 13 persons.

For all our visitors--both domestic and foreign--we express the hope that your visit to New Orleans and participation in this Conference will be rewarding and enjoyable.

I am confident our conference will be successful. There are many favorable factors, including important topics, excellent research to be described by interesting speakers, splendid audience, and unusually good arrangements.

The good arrangements and excellent program did not just happen. I wish to express gratitude to everyone contributing to the conference, including Dr. Aaron M. Altschul, who suggested it and who is General Chairman, Mr. E. L. Patton, Cochairman, Mr. L. W. Mazzeno, Jr., and Mrs. Bea A. Sharar.

However, there is one disappointment. We are regretful because some individuals, scheduled to attend, found that circumstances would prevent their coming to New Orleans. Two of these are: Dr. G. E. Hilbert, Director of USDA's Foreign Research Grant Program, and Dr. George W. Irving, Jr., Deputy Administrator of ARS.

Dr. Irving, formerly an employee of the Southern Division and now in charge of all utilization research and development in USDA, was scheduled to make the opening remarks. For the sake of the conference, I regret he cannot do this. I am honored because he asked me to present his remarks in his absence.



## OPENING REMARKS

George W. Irving, Jr.<sup>1/</sup>  
Deputy Administrator  
Agricultural Research Service  
U. S. Department of Agriculture  
Washington, D. C.

Ladies and Gentlemen: I would like to join Dr. Fisher and others in welcoming you to this Seed Protein Conference. I am happy that so many of you came to participate, and to add a wealth of information on seed protein. I hope that information developed in this meeting will give encouragement to interested workers and organizations in other countries and in the USA in the pursuit of their research on seed proteins. I have an intense interest in this field since some of my earlier work was directly concerned with proteins.

The U. S. Department of Agriculture has had a strong research interest in proteins and for obvious reasons. The early pioneering work on seed proteins was done by scientists like Osborne and Vickery, who were connected with the Connecticut Agricultural Experiment Station, and by D. Breese Jones of the Department at Beltsville. Utilization Research scientists have had a strong interest in seed proteins from the beginning of the establishment of the four Regional Laboratories. A considerable part of the early work on the proteins of cereals, soybeans, peanuts, and cottonseed was done in the Regional Laboratories. I can well remember my own experiences with the proteins of peanuts and with the protolytic enzyme which we found in the resting peanut seed. The reasons for our interest are manifold. The proteins in wheat have a

---

<sup>1/</sup> Presented by C. H. Fisher

profound influence on the structural properties of bread. In the proteins of seed are the major source of protein for animals and man. For these reasons, much of our research has dealt with the physical properties of these proteins as they might affect their utilization, and with properties as they would affect the nutritional value of concentrates and isolates.

It was obvious to all that substantial progress in this field must require fundamental research on the proteins as they exist in the seed without regard to immediate application of the resulting information. Therefore, it is noteworthy that when ARS strengthened its fundamental research by organizing pioneering laboratories some four years ago, as requested by Dr. B. T. Shaw, ARS Administrator, one laboratory (here in New Orleans) was devoted entirely to the study of seed proteins and another, the allergen laboratory, dealt with problems closely related to seed proteins. Other ARS pioneering laboratories are directly involved in protein and plant enzymes research. In these laboratories the scientists have been encouraged to deal with the problems in their most fundamental sense with regard only to methods and approaches that would make it easier to understand these proteins better. It is, therefore, appropriate that this conference--probably the first international meeting devoted exclusively to seed proteins--is being held in one of the laboratories of utilization research, and that the Head of the Seed Protein Pioneering Laboratory, Aaron Altschul, should be Chairman of the meeting.

Our fundamental research, however, is not confined to the pioneering laboratories, and as you will notice in the program, as the meeting

progresses, a number of our other scientists in the Regional Laboratories are dealing fundamentally with the proteins of cereals and of soybeans.

In addition, fundamental research is underway in various foreign institutions with P. L. 480 funds. We are pleased because some of the research leaders in these institutions are with us today.

We could speak at length about the importance of proteins in food over the entire world. Careful and detailed plans for attacking the protein deficit problem on a world basis have been developed by eminent scientists under the sponsorship of UNICEF. We are pleased because Dr. Max Milner of UNICEF is with us attending the meeting. Much work has already been accomplished under these plans. To avoid duplication of effort, careful integration of goals and plans have been maintained. Other organizations active in this area include the FAS and the Secretary's Committee on Food Processing for Developing Nations.

While the work of our Seed Protein Pioneering Research Laboratory in the Southern Division is most important, I would like to mention that utilization research of other types is done. The crops studied in the Southern Division include cotton, cottonseed, tung, sugar, rice, naval stores, cucumbers, citrus, fruits, and miscellaneous vegetables, peanuts, and sweetpotatoes. This work involves the use of scientists of many disciplines. The Division has been fruitful in its research achievements. It has contributed to nearly 200 commercialized developments that have important benefits of retaining old, or creating new, uses for farm products, providing industry with new and profitable products and processes, and giving the consumer new and improved products, sometimes at a lower cost.



The developments aiding industry directly may be referred to as research developments of direct benefit. Agricultural utilization research has been of direct benefit, and in addition has added greatly to our total sum of scientific information about the composition, chemistry and processing of farm crops. This information has stimulated and aided research and development in numerous other research organizations. These "indirect" benefits probably exceed in value the direct contributions of agricultural utilization research.

It is hoped that many of you in attendance at this meeting, and all of you that have a keen interest in this work, will avail yourselves of the opportunity of visiting the Southern Regional Research Laboratory on the Lakefront to meet additional personnel on Dr. Fisher's staff and see the facilities that are used for the protein and other research in progress. As your travel carries you to other parts of the United States, please feel free to visit our other Regional Laboratories in the East, North, and West. I wish to congratulate the Southern Division and to commend Drs. Fisher, Altschul, Patton and others responsible for formulating the program of the caliber that has been arranged. I hope that this will be the beginning of a more organized interest in this very important field of proteins and that as the discussions progress, instances will be found where the study of seed proteins contributes to a general understanding of protein chemistry. I hope that each one of you will feel that the benefit derived from your attendance at this Conference will far outweigh your time and effort in attending.

Thank you.

## SEED PROTEINS -- A PERSPECTIVE

Aaron M. Altschul  
Southern Utilization Research and Development Division  
New Orleans, Louisiana

Before we begin our sessions, I suppose that we ought to inquire about the rationale, if any, for segmenting the field of proteins into one relating particularly to seeds. Perhaps the best rationale lies in a topic which will not be discussed at all at this meeting - the role of seed proteins in human and animal nutrition. There are many in the audience whose major field of interest is protein nutrition; perhaps a word of explanation is due them for not including this area among the subjects to be discussed. Seed proteins are unquestionably the major source of protein for animal and man. As such they deserve major attention. We shall touch on this matter briefly at the luncheon tomorrow. Indeed, the nutritional viewpoint has dominated most of the research on these proteins to date.

If we are, however, to understand these proteins and build a solid basis for dealing with them in whatever application, we must understand them in the environment wherein they exist, in the seed itself. This is where we find them; from here we isolate them; the key to their biochemistry lies in the understanding of the relationship of the proteins to the other constituents and events in the seed. Therefore, it was felt that more fruitful discussions on the seed proteins could take place in a framework of protein chemistry and biochemistry, with the confidence that associated disciplines would derive benefits from progress in this area and would be in a better position to move ahead, as the basic field itself advances.

And so we might look for other rationales for studying seed proteins. These have been pointed out by Tim Anson in several private conversations. The seed could be important as a special biological model of protein and protein synthesis, and seed proteins might serve as models for studying certain problems in protein structure and chemistry. Some seeds synthesize large quantities of proteins, up to 40% of the weight of the kernel in the soybean, for example. Here is a system for synthesis and accumulation of protein that might assist development of our understanding of the mechanism of protein synthesis. Seed storage organs present a complete dichotomy between synthetic and catabolic processes. Compare, for example, fat mobilization in animal adipose tissue and its concomitant reversible synthesis to the two rather cleanly-separated phenomena in a seed such as the castor bean where the synthesis is in the developing bean and the breakdown in the germinating seed.

Seed proteins themselves have special properties which may come from special function on a tissue or particle level. Seed proteins, for example, seem to be highly interacting and more open compared to better organized proteins which are the ones more often studied.

Therefore, we might expect that a study of the proteins and their metabolism would provide information of general value to protein chemistry and biochemistry. Indeed this has happened in the earlier history of protein chemistry. Many of the amino acids were first discovered from seed and plant tissue. Some of the earlier protein techniques were developed on seed proteins. Edestin from hempseed has a long and glorious history as a crystalline protein, easy to obtain. Urease, the first crystalline enzyme, is a seed protein. There is no reason to believe that another



round of study on seed proteins on a more sophisticated level might not be equally productive.

In framing the program of this meeting we had several groups of questions in mind that we hoped might be touched upon in the presentations and discussions. I shall list them to provide you with our own perspective:

1. What is the impact of improvements in protein methodology on the specific problems of purifying and studying seed proteins? Several of the papers will describe examples of application of these techniques to seed proteins and there will also be some general papers describing some of the newer possibilities in protein methodology.

2. What is the role of major proteins in seeds of high protein content? The name of reserve protein has been given to globulins of such seeds. Are they truly reserves, or are they involved in the synthesis of other reserves such as fat, carbohydrate, or phosphate? Are they involved also in the assembly of reserve packages? Where are the proteins in storage tissue? With what kind of organelles are they associated, if any, and what is their metabolism on germination?

3. One might ask about the origin of these proteins. Is there anything that we can say about the manner of accumulation of these proteins compared to what takes place in cells which secrete proteins? Are the proteins vestiges of the enzyme systems of the developing seed, or of mitochondria, or of ribosomes; or are there special synthetic mechanisms for these proteins?

4. What is the relationship of these proteins to the enzyme systems which evolve on germination? Are the precursors for these enzymes from among these proteins or are new enzymes synthesized at another site?

Perhaps we will begin to see some answers as the meeting progresses, or perhaps we will find that these are not the correct questions and that more sophisticated questions must be propounded.

We hope that all of you will find this a fruitful and provocative meeting, as well as an enjoyable one.



# PRINCIPAL PROPERTIES OF WHEAT ALBUMINS

Charles C. Nimmo  
Western Utilization Research and Development Division  
Albany, California

## ABSTRACT

The long history of research on the albumins of wheat has been summarized up to 1944 by C. H. Bailey (1944). Investigations up to that time were almost entirely carried out by extraction with aqueous solution of various salts, with subsequent fractionation by precipitation between salt concentrations to obtain the desired product. Application of electrophoretic and chromatographic methods developed since the late 1930's has shown the heterogeneity of wheat endosperm albumin prepared in this way. In 1953, evidence was presented (2, 3), using filter paper electrophoresis, of the multicomponent character of a wheat flour albumin preparation which had been carefully defined by solubility methods. Despite the heterogeneity of this preparation, there was a consistent pattern of tryptophane and amide-nitrogen content which differentiated similar albumin preparations from globulin preparations for several wheat flour types (4, 5).

Attempts to resolve the albumin proteins have been made by preparative electrophoresis (6) and by column chromatography (7). Only partial separations were obtained, but information on amino acid composition and electrophoretic mobility (free boundary) was obtained. Five fractions derived by preparative electrophoresis (6) contained 9 to 11 percent polysaccharide, indicating definite association between protein and

carbohydrate. Support for this view comes from subsequent other work (8, 9). Evidently either protein or carbohydrate can be the major component of these complexes in wheat flour (10, 11, 12, 13). In connection with research on barley albumin, an alanyl-xylopyranose has been found under conditions which might indicate its function as a protein-carbohydrate bridge (14). Although the protein-carbohydrate complexes can be dissociated by various means (2, 8, 9), their existence in extracted material appears to be definite.

Physical properties of wheat albumins have been studied to a certain extent; values may be revised as components are better separated. Sedimentation constant values for albumin have been reported at 2. to 2.6 by three investigators (2, 15, 16), although heterogeneity is evident. Isoelectric points were determined (2) to range from 4.5 to 8.7 for detectable individual components in the mixture. Approximate electrophoretic mobilities are available in several reports, but variation in pH and other buffer conditions, and inability to cross-identify components make this difficult to summarize.

Recent work which must be related to any discussion of wheat albumins attempted to characterize the soluble protein complex of flour without using classical separations or definitions. Starch gel electrophoresis (pH 3 to 4), with and without urea, was conducted on extracts made with water and aqueous solutions containing salts, acetic acid, aluminum lactate, or alcohol. Comparative separation and electrophoresis indicated that the fastest migrating (cathodic) components contain globulins and that albumins and gliadins are respectively slower (17, 18, 19, 20). Highest component

count reported for a water extract is 22, and for a salt extract, 23 (20). At present a definite figure for the number of non-gluten components cannot be given.

An important series of papers (15, 8, 9) has given basic information on water-extractable proteins (of bleached cake flour) with associated carbohydrate and mineral constituents which may influence protein behavior. Single water extracts of flour (2:1 water:flour) contained about 20% of the flour protein. About one-half the extracted protein was gliadin and one-half albumin. The extract also contained pentosans in about the same amount as the albumins. Gliadin solubility was depressed by dilute salt solutions to reduce the protein yield to about 10% of the flour protein. Electrophoresis (free boundary) showed that all detectable water-soluble components moved to the anode at pH 7.0 or 8.5, and to the cathode at pH 3.8. Curtain electrophoresis (pH 2.3) was used to separate the water-soluble materials into carbohydrate, protein and mineral components. Proteins separated in this way contained not only polysaccharide material, but considerable mono-, di-, and oligosaccharide material (9). The more cationic protein constituents contained substantial quantities of metal ions, and separation was made by re-fractionation at pH 4.1. This detailed approach to the determination of non-protein constituents and their effect on apparent protein properties seems particularly valuable to the understanding of these complex systems.

Fractionation of extracts by chromatography on ion-exchange cellulose (DEAE) has given a considerable concentration of components (16,21), although there is yet no good evidence for homogeneity of any of these.



A considerable portion of extracted material applied to the ion-exchange column was unadsorbed. It appears to consist of several components which have the greatest net positive charge at pH 6 and above (21). The protein of this unadsorbed material was shown to have a higher amide and glutamic acid content, approaching that of gluten, in marked contrast to the amino acid constitution of the proteins fractionated on the anion exchange cellulose (16). Amino acid analyses for various fractions separated by this means are available in both discussions. The proteins adsorbed and fractionated by the ion-exchange showed relatively small differences in amino acid content. There is about five times as much lysine in soluble proteins of this type as there is in gluten. This may be of interest nutritionally, since lysine is the amino acid deficiency of most importance in wheat.

Electrophoresis of water-soluble materials at pH 6 on polyacrylamide gel has shown 15-17 components (21). This means has been used to follow fractionation on DEAE-cellulose as mentioned above and to determine effect of a few pre-treatments of the flour on the pattern of components extracted by water and salt solutions. Neither prior extraction with water-saturated butanol, to remove lipids, nor inclusion of N-ethyl maleimide in the extraction medium (to stabilize sulfhydryl) appeared to have any effect on the pattern. Differences among first, second and third extractions with water were evident, the first extraction showing a much more complex pattern. This led to choice of a single extraction for our work, and a similar idea may perhaps be inferred from other work (15, 8, 9). Location of amylolytic activity among the components has been

demonstrated directly on the gel after electrophoresis (21). It appears to be among the constituents moving most rapidly to the anode; this conclusion has been supported by  $\beta$ -amylase activity determinations on material fractionated by means of DEAE-cellulose.

Immuno-electrophoretic techniques are being used (22) for investigation of wheat proteins. This approach is discussed by another speaker at this conference.

### References

1. Bailey, C. H. The constituents of wheat and wheat products. Reinhold, New York (1944)
2. Pence, J. W. and Elder, A. H. The albumin and globulin proteins of wheat. *Cer. Chem.* 30 (1953) 275-287
3. Pence, J. W., Weinstein, N. E. and Mecham, D. K. Differences in the distribution of components in albumin preparations from durum and common wheat flours. *Cer. Chem.* 36 (1954) 396-406
4. Pence, J. W., Weinstein, N. E. and Mecham, D. K. A method for the quantitative determination of albumins and globulins in wheat flour. *Cer. Chem.* 31 (1954) 29-37
5. Pence, J. W., Weinstein, N. E., and Mecham, D. K. The albumin and globulin contents of wheat flour and their relationship to protein quality. *Cer. Chem.* 31 (1954) 303-311
6. Waldschmidt-Leitz, E., and Hochstrasser, K. Mitteilung über Samenproteine. VII. Über die Albumine aus Gerste und Weizen. *Z. physiol. Chemie.* 324 (1961) 250-253
7. Zueva, E. S., Markosyan, L. S., and Proskuryakov, N. I. Protein chromatography on calcium phosphate gel. *Biokhimiya*, 26 (1961) 209-211. English translation 26 (1961) 187-189
8. Strobel, R. G. and Holme, J. Curtin electrophoretic separation of the water-soluble constituents of bleached cake flour. *Cer. Chem.* (in press)
9. Strobel, R. G. and Holme, J. Chemical compositions of the fraction of the water-solubles of bleached cake flour. *Cer. Chem.* (in press)
10. Neukom, H., Kuendig, W. and Deuel, H. The soluble wheat flour pentosans. *Cereal Science Today*, 7 (1962) 112-114, 125.
11. Neukom, H., Heri, W., Kuendig, W. and Deuel, H. Chromatographische Fraktionierung von Polysacchariden an Cellulose-Anionenaustauschern. *Helv. chim. acta* 43 (1960) 64-71

12. Kuendig, W., Neukom, H. and Deuel, H. Untersuchungen über Getreideschleimstoffe. I. Chromatographische Fraktionierung von Wasserlöslichen Weizenmehlpentosanen an Diäthylaminoäthyl-cellulose. *Helv. chim. acta* 44 (1961) 823-829
13. Kuendig, W., Neukom, H., and Deuel, H. Untersuchungen über Getreideschleimstoffe. II. Über die Gelierung wässriger Lösungen von Weizenmehlpentosanen durch oxydationsmittel. *Helv. chim. acta* 44 (1961) 969-976.
14. Hochstrasser, Karl. Mitteilung über Samenproteine VIII. Über die Bindungsart von Kohlenhydrat and Protein in Gerstenalbumin. *Z. physiol. Chem.* 324 (1961) 250-253
15. Holme, J. Characterization studies on the soluble proteins and pentosans of cake flour. *Cer. Chem.* 39 (1962) 132-144
16. Coates, J. H., and Simmonds, D. H. Proteins of wheat and flour. Extraction, fractionation and chromatography of the buffer-soluble proteins of flour. *Cer. Chem.* 38 (1961) 256-272
17. Woychik, J. H., Boundy, J. A., and Dimler, R. J. Starch gel electrophoresis of wheat gluten proteins with concentrated urea. *Arch. Biochem. Biophysics.* 94 (1961) 477-482
18. Elton, G. A. H., and Ewart, J. A. D. Starch gel electrophoresis of wheat proteins. *Nature* 187 (1960) 600-601, *J. Sci. Food Agric.* 13 (1962) 62-72
19. Coulson, C. B. and Sim, A. K. Starch gel electrophoresis of isolated wheat gluten. *Biochem. J.* 80 (1961) 46-47
20. Kaminski, E. Study of wheat proteins soluble in water, salt solution, 70% ethanol and dilute acetic acid by starch-gel electrophoresis. *J. Sci. Food Agric.* 13 (1962) 603-607
21. Nimmo, C. C., O'Sullivan, M. T., Mohammad, A. and Pence, J. W. Fractionation and zone electrophoresis of proteins of water-soluble materials of flour. *Cer. Chem.*, in press.
22. Grabar, P., Benhamou, N. and Daussant, J. Etudes électrophorétiques et immunochimiques de quelques protéines de l'orge et du blé. *Arch. Biochem. Biophys. Supplement* 1 (1962) 187-199



# STRUCTURAL INVESTIGATIONS OF WHEAT GLIADIN AND GLUTENIN<sup>1</sup>

John H. Woychik

Northern Regional Research Laboratory,<sup>2</sup> Peoria, Illinois

Wheat gluten is unique among plant proteins primarily because of its cohesiveness, extensibility, and elasticity when hydrated. These properties enable gluten proteins to hold a bread dough together and to retain gas produced by fermentation during the baking process, to give baked goods their characteristic cellular structure. These unique properties have prompted numerous investigations designed to explain the chemical and physical structures responsible.

Wheat gluten can be separated into the 70% ethanol-soluble prolamine, gliadin, and into the alcohol-insoluble fraction, glutenin. These fractions account for about 80% of the endosperm protein of the wheat kernel. The protein makeup of these fractions is shown in Figure 1. The alcoholic fractionation has separated the migrating components, which comprise the gliadin fraction (left pattern), from the glutenin protein, which is retained at the gel origin (right pattern). This retention of the glutenin fraction at the point of insertion into the gel has been attributed to the large molecular size and possibly to a particular configuration that prevents the protein molecule from migrating through the starch gel pores<sup>(1)</sup>. Glutenin, however, does migrate in moving boundary electrophoresis as a single broad peak.

---

<sup>1</sup> Presented at the Seed Protein Conference, New Orleans, La., January 21-23, 1963.

<sup>2</sup> This is a laboratory of the Northern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture.

Starch gel electrophoresis of gliadin resolves this fraction into 7 major components and up to 10 or more minor components. Patterns of gliadins from different wheat varieties vary from that in Figure 1. The gliadin fraction has a weight average molecular weight of approximately 40,000 in aluminum lactate buffer, whereas glutenin exhibits a spectrum of sedimenting species with a weight average molecular weight of 2 to 3 million<sup>(3)</sup>.

Finding a high molecular weight for the glutenin fraction fits well with the observation of its failure to migrate into the starch gel. Cleavage of the disulfide bonds of glutenin, either by performic acid oxidation or by reaction with sulfite, reduces it to ultracentrifugally homogeneous species with a molecular weight of about 20,000, with an attendant loss of elastic and cohesive properties<sup>(4)</sup>. This finding supports a concept of glutenin formation through extensive inter-molecular disulfide bonding of comparatively low molecular weight components. Such a mechanism would also permit the incorporation of gliadin or water-soluble proteins into the glutenin structure.

In view of the marked effect of reduction upon the molecular weight and physical properties of glutenin, it was desirable to investigate this effect on its electrophoretic behavior. The effect of reduction on the gliadin fraction was also studied. Several methods exist for the cleavage of disulfide bonds in protein. However, not all are readily applicable to proteins on which further structural studies are contemplated. The presence of appreciable amounts of tryptophan in gluten proteins warrants the exclusion of oxidative techniques; similarly the alkaline lability of gluten sulfur demands the use of relatively gentle reductive techniques.



Mercaptoethanol reduction at pH 8 in 8 M urea was chosen for reduction of gluten proteins. After the liberation of sulfhydryl groups by reductive techniques, an alkylation procedure normally is used to prevent reoxidation of the liberated groups. In some instances however, alkylation can produce artifacts through nonspecific reactions with amino groups and with methionine sulfur. Therefore electrophoretic patterns were first obtained of reduced nonalkylated gliadin and glutenin, which could be subsequently used for comparisons with their alkylated counterparts. The electrophoretic patterns of gliadin and glutenin after reduction with a 50 M excess of mercaptoethanol are shown in Figure 2.

The reduced state of the two fractions was maintained by including 0.05 M mercaptoethanol in the starch gel and buffer system. Varying the reduction time from 15 minutes to 24 hours did not result in electrophoretic patterns different from those of Figure 2. Figure 3 presents a schematic diagram obtained from a compilation of several patterns of the reduced proteins to define more clearly the number and relative intensities of electrophoretic components. The gliadin fraction (A) showed no increase in the number of major components following reduction; seven principal bands were present in the native protein and in the reduced protein. The gliadin components appear to have been altered structurally, however, as evidenced by a retardation of approximately 20% in their electrophoretic mobilities compared to those of the native protein.

The failure to observe an increase in the number of electrophoretic components in reduced gliadin leads to the tentative conclusion that these proteins consist of a single-chain polypeptides containing intramolecular

disulfide bonds. Reduction of glutenin (B) released 20 or more components from a protein that showed no gel migration in the native state. Over half of these components are present only in trace amounts and have mobilities greater than the fastest gliadin component. The major portion of the glutenin protein can be accounted for by the six principal components shown in Figures 2 and 3.

A comparison of electrophoretic patterns in Figure 3 indicates that all components in reduced glutenin have counterparts with identical mobilities in reduced gliadin. The exceptions are trace components that have electrophoretic mobilities greater than the fastest gliadin component. These trace components, not shown in the figure, probably originate from incorporated components comparable to, or identical with, water-soluble proteins of wheat, which have relatively high electrophoretic mobilities. Although major differences in protein distribution are evident among the components of reduced gliadin and reduced glutenin, finding apparently identical components within these fractions suggests that glutenin arises primarily through intermolecular disulfide bonding of gliadin components. The difference in distribution could result from several causes, either selectivity in the formation of intermolecular disulfide bonds or the presence of a different distribution of gliadin components at the time or site of glutenin synthesis. Water-soluble proteins are probably also incorporated into the glutenin structure although to a far lesser degree.

Further investigations necessitated preparation of reduced protein in a form in which the liberated sulfhydryl groups were protected from reoxidation. Iodoacetic acid and its amide have been widely used as alkylating agents. In addition to these, Weil and Seibles<sup>(5)</sup> reported

using acrylonitrile for stabilizing reduced lactalbumin and lactoglobulin to yield cyanoethylated derivatives. Comparison of electrophoretic patterns revealed that components with significantly greater electrophoretic mobilities were obtained after alkylation of glutenin with iodoacetamide than with acrylonitrile, although the number of components in each case appeared identical. When cyanoglutenin was compared directly with the reduced nonalkylated glutenin, no differences were observed either in the electrophoretic mobilities or in the number of components. The increased mobility of components in iodoacetamide treated glutenin probably result from an increased net positive charge arising from the reaction of iodoacetamide with methionine sulfur to yield a methionine sulfonium compound<sup>(6)</sup>. On the basis of these observations, we conclude that acrylonitrile is better suited for the alkylation of reduced gluten proteins.

The presence of electrophoretic components with identical mobilities in reduced gliadin and reduced glutenin does not in itself confirm the idea that glutenin arises through intermolecular disulfide bonding of gliadin and possibly of water-soluble protein. Such proof requires a demonstration of identical amino acid composition or peptide maps from components isolated from gliadin and glutenin. However, some supporting evidence for this hypothesis was provided by the determination of N-terminal residues in these proteins. The amino acids found to occupy N-terminal positions in these protein fractions are presented in Table 1. Data for water-soluble proteins are also included. The principal N-terminal residues in gliadin and glutenin were aspartic acid, glutamic acid, and serine. Substantially lesser amounts of threonine, and alanine occurred



in gliadin but none in glutenin. The N-terminal residues of the water-soluble proteins were identical with those of gliadin. The inability to detect threonine, and alanine in glutenin may arise because these residues originate from reduced gliadin components having only trace counterparts in reduced glutenin.

Amino acid analysis of three chromatographic fractions from reduced and cyanoethylated glutenin revealed similar distributions with principal differences in basic amino acid content. In each of these fractions, approximately 40% of the residues were accounted for by glutamine and about 15% by proline. The high content of glutamine and proline suggests the possibility of some rather unique structural sequences in these proteins.

Our investigation of gliadin proteins have centered primarily about one of these components. Through a combination of ion-exchange chromatography and continuous-flow curtain electrophoresis, we were able to obtain sufficient amounts of the gamma-gliadin component to pursue further investigations. This component was selected for detailed study because it probably represents the structural makeup of almost all gluten proteins. The amino acid composition of gamma gliadin in moles per 25,000 grams is presented in Table 2. Glutamine accounted for approximately 40% of the residues; proline 20%.

A minimum molecular weight of 25,000 was calculated for gamma gliadin from the amino acid data. The molecular weight of gamma gliadin was determined in several solvent systems from sedimentation data treated by the method of Trautman<sup>(7)</sup>. In an aluminum lactate buffer at pH 3.1, a value of approximately 50,000 was obtained for native protein; however,

the reduced protein showed extensive aggregation with molecular weights ranging up to several million. In 4 M guanidine hydrochloride both native and reduced proteins had a molecular weight of 26,000, a figure in good agreement with the calculated value. The failure to observe a decreased molecular weight after reduction suggests a single-chain structure. This idea is supported by finding a single N-terminal residue, aspartic acid, and one C-terminal residue, serine.

By using reduction studies on starch gel electrophoresis we have tentatively established that the glutenin fraction, which appears to be the primary factor responsible for the rheological properties of gluten protein, arises through intermolecular disulfide bonding of gliadin and water-soluble protein components. Finding the same amino acids in the N-terminal positions in both gliadin and glutenin tends to support this hypothesis. Amino acid analysis of chromatographic fractions of reduced glutenin revealed patterns not too dissimilar from those of corresponding gliadin components. Detailed structural investigations are now centered upon the gamma-gliadin component, with the hope of understanding better the relationship between structure and properties of gluten proteins.



## REFERENCES

1. J. H. Woychik, J. A. Boundy, and R. J. Dimler, Arch. Biochem. Biophys. 94, 477 (1961).
2. O. Smithies, Biochem. J. 61, 629 (1955).
3. R. W. Jones, G. E. Babcock, N. W. Taylor, and F. R. Senti, Arch. Biochem. Biophys. 94, 483 (1961).
4. H. C. Nielsen, G. E. Babcock, and F. R. Senti, Arch. Biochem. Biophys. 96, 252 (1962).
5. L. Weil and T. S. Seibles, Arch. Biochem. Biophys. 95, 470 (1961).
6. H. G. Gundlach, S. Moore, and W. H. Stein, J. Biol. Chem. 234, 1761 (1959).
7. R. Trautman, J. Phys. Chem. 60, 1211 (1956).

Table 1.--N-Terminal Amino Acids of Protein Fractions

<u>Gliadin</u>	<u>Glutenin</u>	<u>Water-solubles</u>
Asp	Asp	Asp
Glu	Glu	Glu
Ser	Ser	Ser
Thr		Thr
Ala		Ala

Table 2.--Amino Acid Composition\* of Gamma Gliadin

IYS 2	PRO 38	LEU 14
HIS 3	GLU 32	TYR 2
NH <sub>3</sub> 84	GLY 6	PHE 10
ARG 3	ALA 7	TRY 1
ASP 4	VAL 9	CYS (1/2) 6
THR 5	MET 2	
SER 10	ILUE 10	

\* Moles/25,000 g.

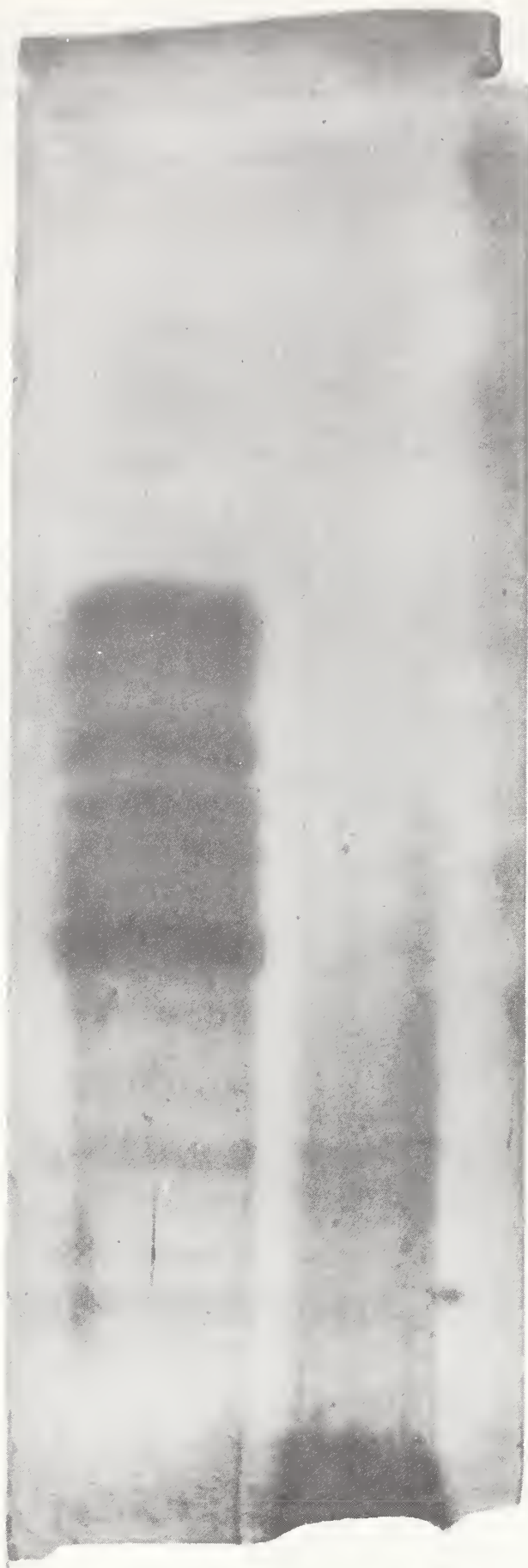
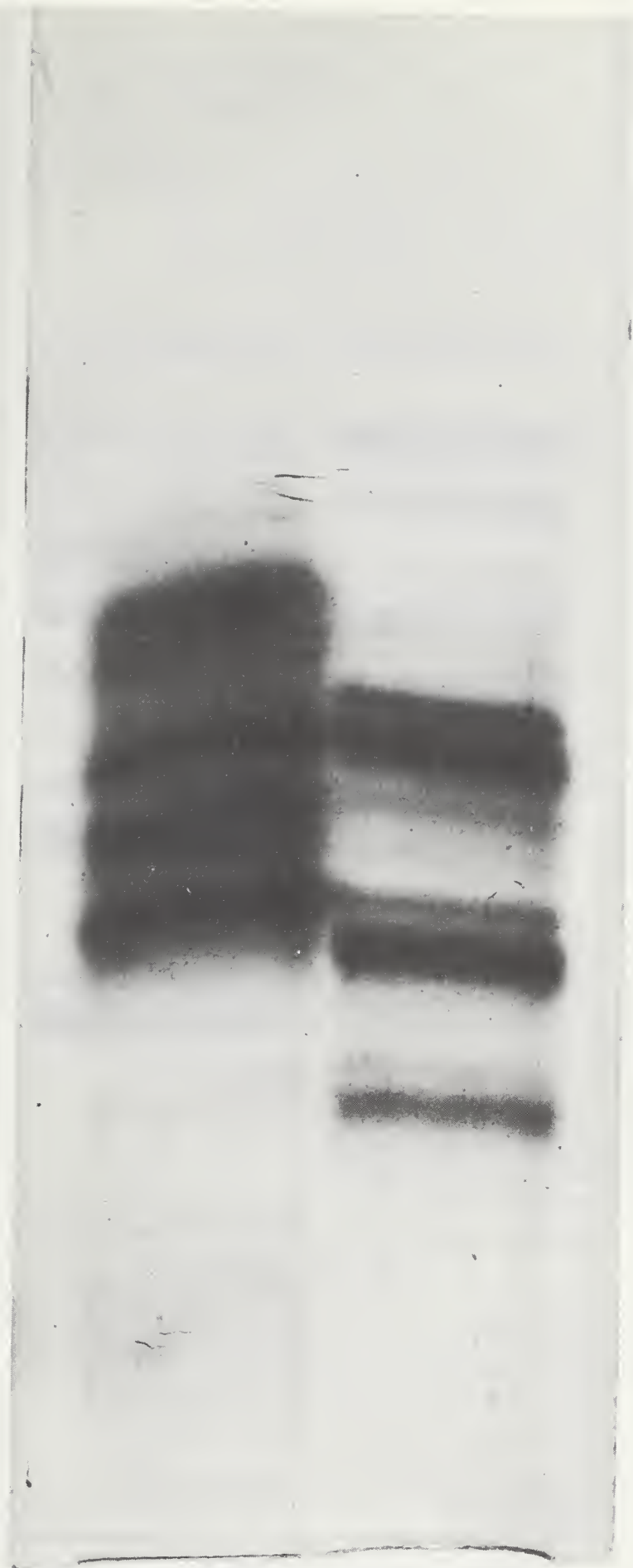


FIGURE 1  
Starch gel electrophoretic patterns of gliadin (left) and glutenin (right).



**A**

**B**

FIGURE 2  
Starch gel electrophoretic patterns of reduced gliadin  
and glutenin (nonalkylated). A-gliadin B-glutenin.



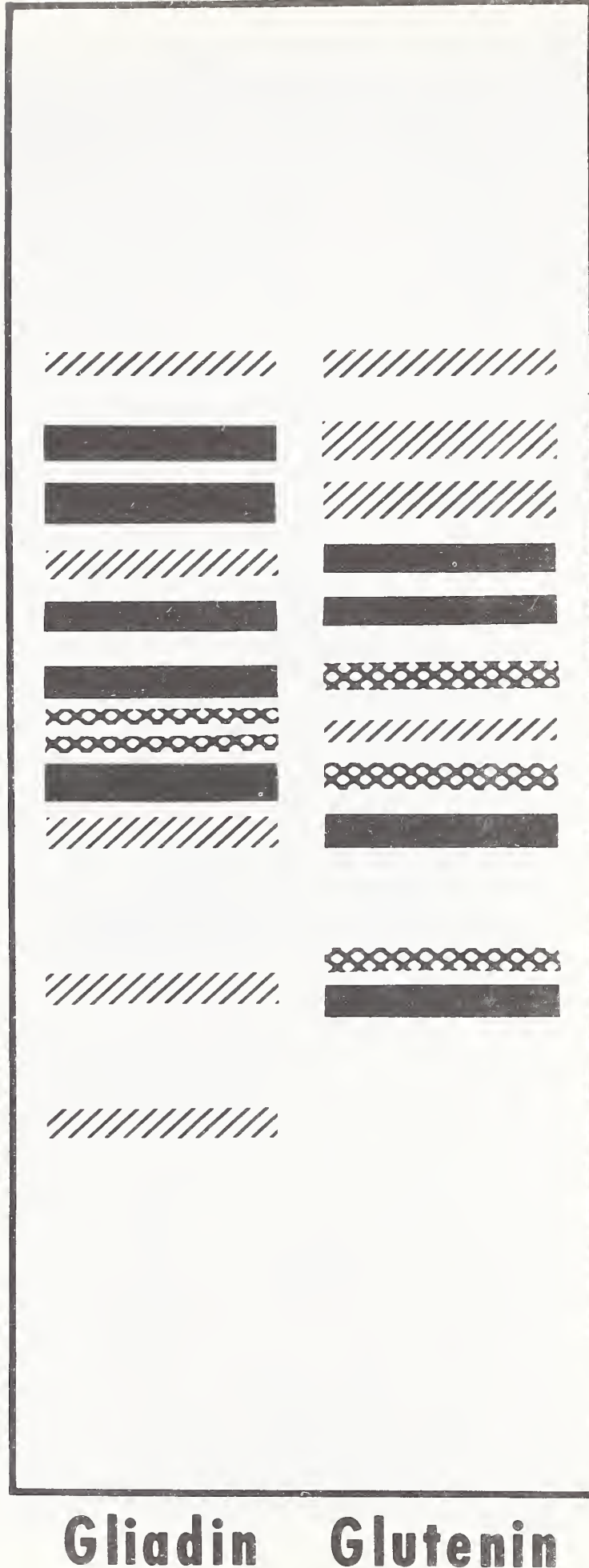


FIGURE 3  
Schematic diagram showing the number and relative intensities of electrophoretic components obtained after reduction of gliadin and glutenin (nonalkylated).

## Discussion

- Dr. Bruno Jirgensons: The high proline content of  $\gamma$ -gliadin is very interesting. Have structure studies (determination of  $\alpha$ -helix) been made?
- Dr. John H. Woychik: Not as yet, however, whole gliadin has a low  $\alpha$ -helix (10-15%).
- Dr. Pierre Grabar: We attempted to study the production of antibodies to gluten, but had solubility problems.
- Dr. Daniel K. O'Leary: Components in gluten are very easily digested by pepsin because of the high glutamine content.
- Dr. Donald N. Duvick: Is glutenin an artifact?
- Dr. John H. Woychik: No, it is a distinct fraction formed at a specific time in the wheat kernel. Glutenin is apparently formed before gliadin (as determined by incorporation of labelled amino acids).
- Dr. George R. Tristram: It was found that some of the N-terminal groups of collagen are really contaminating amino acids. Some of Woychik's end groups may be due to contaminating amino acids or small peptides.
- Dr. John H. Woychik: The 1-1 relationship between the N-terminal and the C-terminal acids found in gamma gliadin precludes the possibility of free amino acids, however, it could be possible in gliadin or glutenin.
- Dr. Bruno Jirgensons: The results often depend on the method used to isolate the protein. The end groups of Woychik's protein are the same as those of  $\gamma$ -globulin. How do molar results compare?
- Dr. John H. Woychik: The DNP-method did not give good recovery.
- Dr. George R. Tristram: The end groups of collagen are also the same as Woychik's protein.

# IMMUNOCHEMICAL STUDIES ON WHEAT AND BARLEY PROTEINS

Pierre Grabar, Nicole Benhamou and Jean Daussant  
Institut Pasteur, Paris

## SUMMARY

Recently, we have published the results which we have obtained in our electrophoretic and immunochemical analysis of "water and salt-soluble proteins" of wheat and barley. 8-10 constituents could be detected in wheat and 17-22 in barley extracts, and their mobilities have been calculated. Various fractionation procedures have been used and some of the sub-fractions could be purified (Arch. Biochem. & Biophys., 1962, suppl. 1, 187-199)

Using the same immunochemical methods, "insoluble" proteins from wheat have been studied. These proteins have been dissolved in acid buffers, or in 3M urea, an immunserum has been prepared and electrophoretic analysis (in urea) has been performed. Immunochemical analysis has shown that such "solubilizations" do not alter the antigenicity of these proteins. Further studies on these proteins are in progress.

Studies on the proteolytic activity of wheat and barley extracts have been undertaken. The optimum pH for the degradation of human serumalbumin has been found to lie between pH 3.5 and 3.8. The degradation products of this protein have been studied using the immunoelectrophoretic analysis and have been compared with products obtained by the action of animal proteases. The presence of proteolytic activity in various sub-fractions of wheat and barley proteins has been investigated; it is particularly strong in fraction  $\alpha_1$  and to a lesser degree in fraction  $\alpha_2$ .

Preliminary studies have shown that some of the water and salt-soluble wheat and barley proteins are antigenically similar; i.e. some of these proteins react with the immune sera against the other extract. Thus they are analogous or even identical. Such studies may furnish interesting information on the genetic origins or relations between wheat and barley.

(These studies are performed with partial financial support by the Agricultural Research Service, U. S. Department of Agriculture.)

#### Discussion

- Dr. Donald C. Abbott: What quantity of protein is necessary to prepare an antibody?
- Dr. Pierre Grabar: About 25 mg./rabbit.
- Dr. George R. Tristram: Have enzymes in the proteins been identified?
- Dr. Pierre Grabar: Enzymatic activity is not destroyed by the antigen-antibody reaction, and enzymes can be identified after immunoelectrophoresis.
- Dr. Pierre Grabar: If a protein is partially denatured, a prolonged line, rather than the regular arc, is obtained on immunoelectrophoresis. The two mobilities of a polymerized material give a double arc.
- Dr. M. L. Anson: The "peakology" resulting from the separation of oilseed proteins can be a source of confusion unless the peaks are properly interpreted. Above all, one wants to know whether two proteins giving two different peaks are close relatives or not relatives at all, and also whether two different proteins from different oilseeds are related or not.



Closely related proteins can give different peaks if they are genetic variants, or if one of the proteins is a compound with some other substance in solution or a result of some chemical modification of the original protein, say by the loss of an amide group.

In case the protein of a given peak has no known enzymatic or other biological activity, the attempt must be made to characterize it by a variety of chemical methods.

Dr. Aaron M. Altschul:

Direct enzymatic activity of all these proteins may not be discovered since some of these proteins may be structural proteins.

Dr. Pierre Grabar:

Analysis of animal cells should yield thousands of enzymes; however, only a few are obtained. These may probably be apo-enzymes, common to several enzymatic activities.

# RECENT ADVANCES IN THE CHEMISTRY OF SOYBEAN PROTEINS<sup>1/</sup>

W. J. Wolf

Northern Regional Research Laboratory,<sup>2/</sup> Peoria, Illinois

The present status of the chemistry of seed proteins in general and soybean proteins in particular is very aptly summed up as:

"The study of the chemistry of plant proteins, although it early interested several of the leading chemists of their time, has received in the aggregate so little attention that today our knowledge of this subject is but slightly advanced beyond what may properly be called the beginning."

Surprisingly, this statement was made 55 years ago by one of the pioneers in this field, Thomas B. Osborne (1). Yet, in comparing the state of our knowledge of plant proteins with that of protein chemistry in general, this statement is still remarkably accurate. The scope of this conference and depth of its program, however, indicate that progress is being made and that interest in research in this area will increase.

The two major topics of soybean protein chemistry I shall discuss are: (a) The question of heterogeneity of glycinin, the major fraction of soybean proteins, and (b) lipid-protein complexes isolated from defatted soybean meal.

---

<sup>1/</sup> To be presented at Seed Protein Conference, New Orleans, La., January 21-23, 1963.

<sup>2/</sup> This is a laboratory of the Northern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture.

## Heterogeneity of Glycinin

Soybean globulins are readily extracted from hexane-defatted meal by either water or salt solutions but are insoluble at their isoelectric point near pH 5 in the absence of salts. Over 60 years ago Osborne and Campbell (2) isolated soybean globulins by extracting soybean meal with a 10% sodium chloride solution, and collecting the fraction that precipitated when the extract was dialyzed against water. They believed that this fraction was a single protein and called it glycinin.

A summary of some of the studies made on glycinin and conclusions regarding its homogeneity appear in Table 1. Although originally regarded as homogeneous, the techniques of electrophoresis and ultracentrifugation indicated the presence of more than one component. Recently Kretovich and coworkers (7) attempted to fractionate glycinin by ammonium sulfate precipitation but were unsuccessful. They then studied the sedimentation behavior of glycinin and observed that the number of ultracentrifuge components observed depended on pH and ionic strength. At low pH only a single component with a sedimentation coefficient of 2-3S and a molecular weight of about 20,000 was detected. As a result of these studies, they proposed the following hypothesis: That glycinin is made up of identical subunits (the 2-3S component), which are capable of reacting with themselves reversibly to form aggregates of various sizes depending upon conditions. Association and dissociation of the identical subunits are proposed to explain the apparent heterogeneity of glycinin. The reactions postulated to be responsible for the four ultracentrifuge components of glycinin at pH 7.6, 0.5 ionic strength, are shown in Figure 1. In Figure 1, 2S



represents the subunit state and the other components represent aggregates of the subunits. This concept, if correct, is very important because it means that attempts to fractionate and purify soybean globulins are unnecessary. It would also be extremely difficult to isolate stable forms of the aggregates under conditions where association-dissociation reactions occur unless the rates of interconversion are very slow.

Soybean protein fractionation studies by other workers indicate that stable fractions of glycinin can be obtained (6, 8). For example, the 11S component, one of the major components of glycinin at pH 7.6, 0.5 ionic strength, is readily obtained in a state of purity of 65-75% by cooling a concentrated aqueous extract of soybean meal. The 11S component in this crude state of purity shows no tendency to revert to the original distribution of the four components observed in glycinin, indicating that a definite fractionation has occurred. More recently we have been able to purify the 11S component to above 90% purity either by ammonium sulfate fractionation or by chromatography on calcium phosphate (9). Figure 2 shows three ultracentrifuge patterns demonstrating the purification attainable by ammonium sulfate fractionation. Figure 3 shows the chromatographic behavior of water-extractable proteins, crude 11S protein (cold-insoluble fraction) and purified 11S protein on calcium phosphate. In water-extractable proteins (Fig. 3) peak A consists of 2S protein, peak B is a mixture of 2S and 7S components, peak C is primarily the 11S and 15S components, and peak D is a 7S component. Chromatography of the cold-insoluble fraction yields one major peak consisting of the 11S and 15S components. Chromatography of the purified 11S component demonstrates its homogeneity; similar behavior was observed on diethyl-aminoethyl



cellulose chromatography (9). The 11S component is, therefore, stable to adsorption and desorption during chromatography. The calcium phosphate chromatography studies also indicated the presence of two 7S fractions in the water-extractable proteins, one in fraction B and one in D.

Additional evidence that the ratio of 7S and 11S components observed in glycinin (Figure 1) does not represent equilibrium concentrations of aggregates of identical subunits is the observation that some Japanese soybeans have 2:1 ratios of 7S to 11S components instead of about 1:1 usually observed in soybean varieties from this country (10). The ratio of 7S to 11S components in the globulin fraction can also be varied by selectively denaturing and insolubilizing the 7S fraction with 50% ethanol (11). The 11S component resists alcohol denaturation more.

The criteria for heterogeneity of glycinin presented are all based on physical properties of the globulin fraction. There is, therefore, a definite need for chemical evidence to settle the question of homogeneity or heterogeneity of glycinin and the presence or absence of identical subunits. The N-terminal amino acid determinations of Campbell, Meyer, and Circle (12), discussed by Wolf and Smith (13), strongly suggest that the subunits of the 11S component are nonidentical. Other legume seed globulins reportedly contain nonidentical subunits (14, 15). Indeed, soybean globulins would be unique if identical subunits were present.

#### Lipid-Protein Complexes

In 1946, Smiley and Smith (16) reported that extraction of soybean globulins with ethanol yielded a brown siruplike material. Upon hydrolysis this material yielded phosphoric acid, fatty acids, and

choline indicating the presence of phospholipids. We have recently confirmed the presence of this lipidlike material and have begun preliminary characterization studies of it. It appears to be a complex mixture.

The presence of these lipidlike materials poses some important questions:

1. Are the lipids contaminants or are they structural elements of the protein molecules, i.e., are they "molecular glues" cementing the protein subunits together?
2. Do these lipid-protein complexes exist in the intact seed?
3. Are the lipids combined with specific proteins or is there a random distribution of lipids among the various proteins?
4. Does removal or addition of lipid cause a shift in the relative amounts of the proteins, such as the 7S and 11S components?
5. Do these lipidlike materials alter the physical properties of the proteins?
6. Is this type of material present in other "purified" seed globulins?
7. Can methods be developed to remove these lipids without causing irreversible conformational changes in the proteins?

The answers to most of these questions are still unknown, but some partial solutions have been obtained in preliminary studies (17). Among the most effective solvents for removal of the lipid material from isolated soybean proteins are aqueous alcohols and aqueous acetone (17). Water appears to be an important component of the solvent mixture and the concentration of water required for optimum extraction of the lipid materials depends upon the kind of alcohol used.

Figure 4 shows the effect of ethanol concentration on the amount of solids and lipids (measured as esters with hydroxamic acid) removed from acid-precipitated protein and also the nitrogen content and foam stability of the protein after extraction with alcohol. Extraction in the range of 78-84% ethanol removes the most solids and lipids, and yields a protein preparation having the highest nitrogen content.

A pronounced change in the physical properties of the proteins after extraction with alcohol is an increased tendency to foam. Aqueous solutions of the alcohol-extracted proteins whip into voluminous foams, which are extremely stable. Maximum foam stability is obtained upon extraction with 86% ethanol (Figure 4).

There is no conclusive evidence yet that the lipids react with specific proteins. On examination of various soybean protein fractions, all contained lipids to varying degrees, except for the 11S protein component purified by ammonium sulfate precipitation (17). It contained only a trace of material extractable with 86% (v/v) ethanol. However, the presence of oxidized lipid tightly bound to the protein has not been ruled out.

Maximum insolubilization of soybean globulins by treatment with aqueous ethanol at room temperature occurs at 50-60% alcohol concentration (11). Under these conditions the 7S fraction accounts for most of the loss in protein solubility. Is the removal of lipid from the 7S fraction responsible for loss of solubility of the 7S fraction? Figure 4 indicates that maximum removal of lipid occurs at much higher alcohol concentrations ( $\sim 80\%$ ), but there exists the possibility that the curve for soluble esters is the summation of the extractability of a number of



lipids and that the lipids extracted with 50% alcohol are associated specifically with the 7S fraction. Maximum extraction of lipids with 86% ethanol occurs at alcohol:protein ratios of 20:1 (ml. alcohol per gram of protein) or greater. At an alcohol:protein ratio of 2:1 only about 40% of the maximum amount is extracted. However, there was no difference in solubility of acid-precipitated protein (at pH 7.6, 0.5 ionic strength) extracted at ratios of 50:1 or 2:1, nor was there any difference in ultracentrifugal composition of the soluble proteins. These results suggest that loss of protein solubility is due to alcohol denaturation rather than to removal of lipid (18). There also was no indication of interconversion of the 7S and 11S components because of removal of the lipids.

Sensitivity of soybean proteins to alcohols indicates that other techniques may have to be devised to remove lipid materials without altering the proteins.

### Summary

Soybean globulins have been fractionated by ammonium sulfate precipitation, by calcium phosphate chromatography, and by selective denaturation with aqueous alcohols. Glycinin appears to be a mixture of different proteins, and the primary problem is to devise methods for separating these proteins from one another. The purification task is further complicated by the presence of phospholipids in isolated proteins. This introduces problems such as lipid oxidation, need for developing methods for removal of lipids, and elucidation of the significance of these lipids in isolated soybean proteins.



## REFERENCES

1. T. B. Osborne, Science 28, 417 (1908).
2. T. B. Osborne and G. F. Campbell, J. Am. Chem. Soc. 20, 419 (1898).
3. D. B. Jones and F. A. Csonka, J. Biol. Chem. 97, XXIX (1932).
4. C. E. Danielsson, Biochem. J. 44, 387 (1949).
5. D. R. Briggs and R. L. Mann, Cereal Chem. 27, 243 (1950).
6. W. E. F. Naismith, Biochem. Biophys. Acta 16, 203 (1955).
7. V. L. Kretovich, T. I. Smirnova, and S. I. Frenkel, Biokhimiya 23, 547 (1958).
8. W. J. Wolf and D. R. Briggs, Arch. Biochem. Biophys. 85, 186 (1959).
9. W. J. Wolf, G. E. Babcock, and A. K. Smith, Arch. Biochem. Biophys. 99, 265 (1962).
10. W. J. Wolf, G. E. Babcock, and A. K. Smith, Nature 191, 1395 (1961).
11. R. Roberts and D. R. Briggs, Abstract of Papers, No. 66, 47th Meeting, AACC, May 1962.
12. B. J. Campbell, E. W. Meyer, and S. J. Circle, Abstract of Papers, 69C, 131st Meeting, ACS, April 1957.
13. W. J. Wolf and A. K. Smith, Food Technol. 15, 12 (1961).
14. C. W. Cater, W. E. F. Naismith, R. H. K. Thomson, and G. R. Ure, Nature 180, 971 (1957).
15. I. A. Vaintraub and Y. Y. Gofman, Biokhimiya 26, 13 (1961).
16. W. G. Smiley and A. K. Smith, Cereal Chem. 23, 288 (1946).
17. A. C. Eldridge, W. J. Wolf, A. M. Nash, and A. K. Smith, Alcohol Washing of Soybean Proteins, J. Food Agr. Chem., In press.
18. W. J. Wolf, A. C. Eldridge and G. E. Babcock, Physical Properties of Alcohol Extracted Soybean Proteins, Cereal Chem., In press.

Table 1.--Summary of Studies on Glycinin

Investigators	Date	Method of Study	Conclusion
Osborne and Campbell (2) <sup>1/</sup>	1898	Solubility behavior and chemical composition	Homogeneous
Jones and Csonka (3)	1932	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> fractionation	Heterogeneous
Danielsson (4)	1949	Ultracentrifuge	Heterogeneous
Briggs and Mann (5)	1950	Electrophoresis	Heterogeneous
Naismith (6)	1955	Ultracentrifuge	Heterogeneous
Kretovich <u>et al.</u> (7)	1958	Ultracentrifuge	Homogeneous
Wolf and Briggs (8)	1959	Ultracentrifuge	Heterogeneous

<sup>1/</sup> Numbers in parentheses are literature references.



## Reversible Equilibrium Reactions:

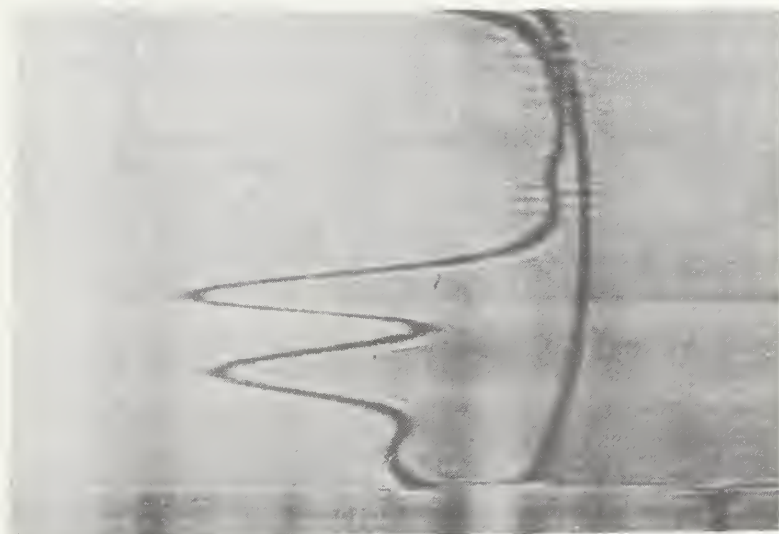


FIGURE 1  
Ultracentrifuge pattern for glycinin at pH 7.6, 0.5 ionic strength and equilibrium reactions proposed by Kretovich et al. (7).

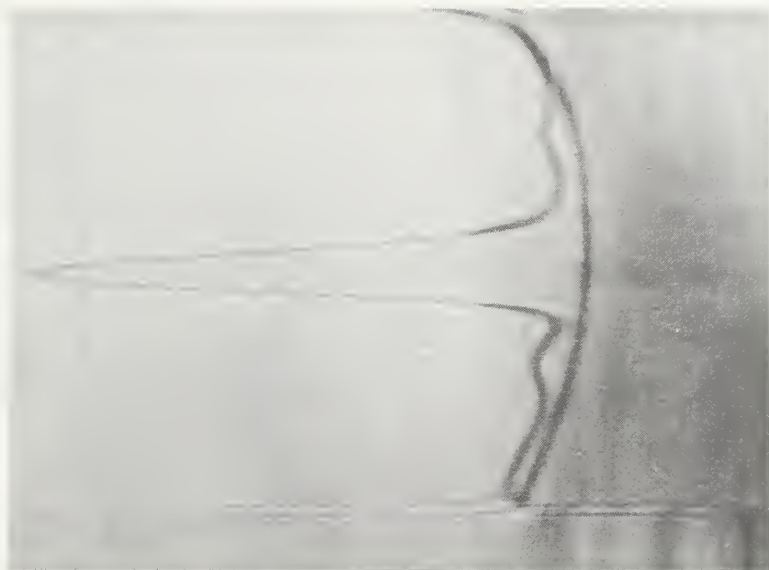
2 7 11 15

11

11



(a)



(b)



(c)

FIGURE 2  
Ultracentrifuge patterns for: (a) water extract of soybean meal, (b) cold-insoluble fraction of soybean protein, and (c) the 11S component obtained from (b) by ammonium sulfate precipitation.



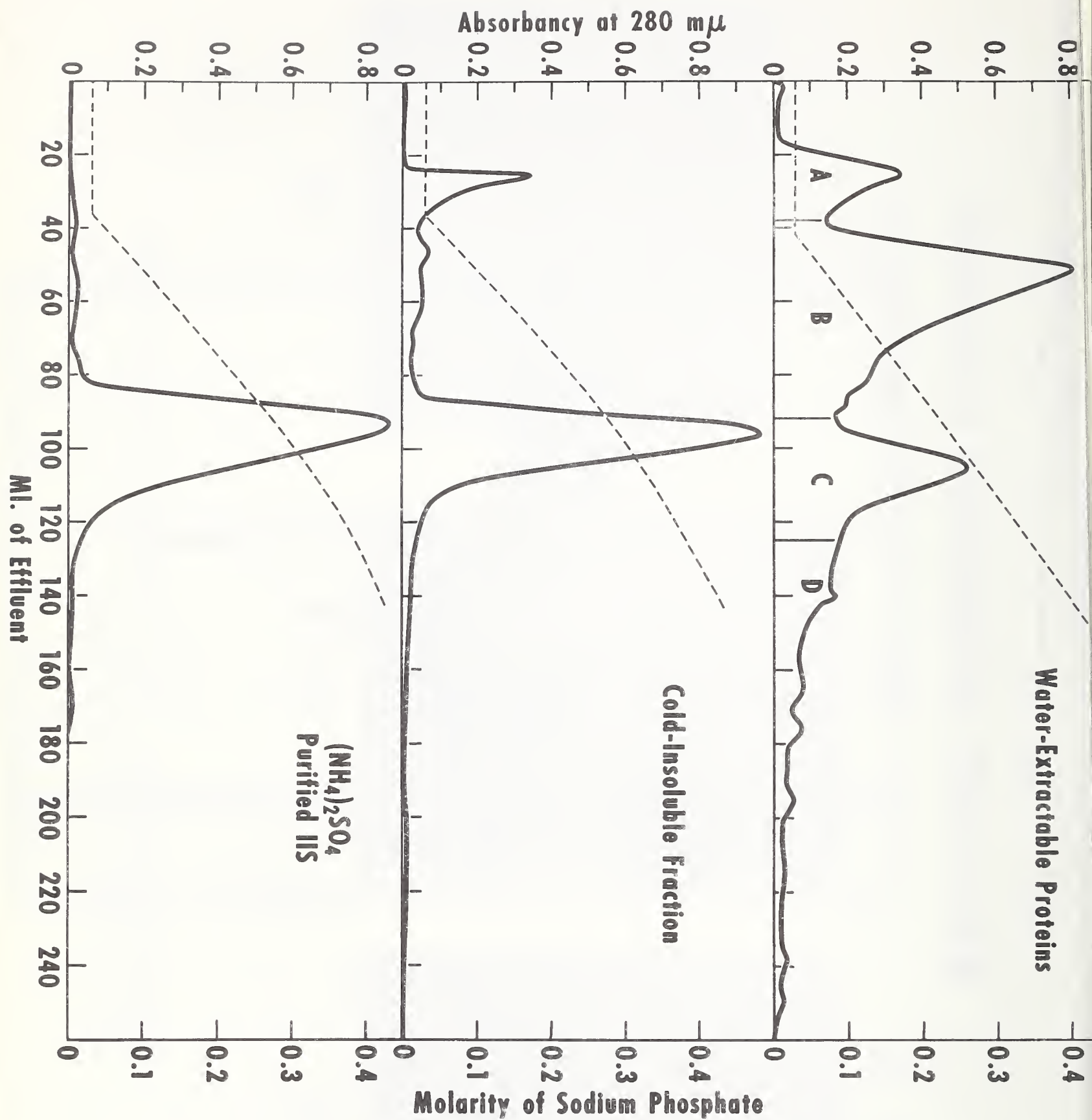


FIGURE 3  
Calcium phosphate chromatography of water-extractable soybean proteins (top), cold-insoluble protein (middle) and IIS component purified by ammonium sulfate precipitation (bottom). Solid curve is absorbance at 280 mμ and dashed curve is the gradient of sodium phosphate.

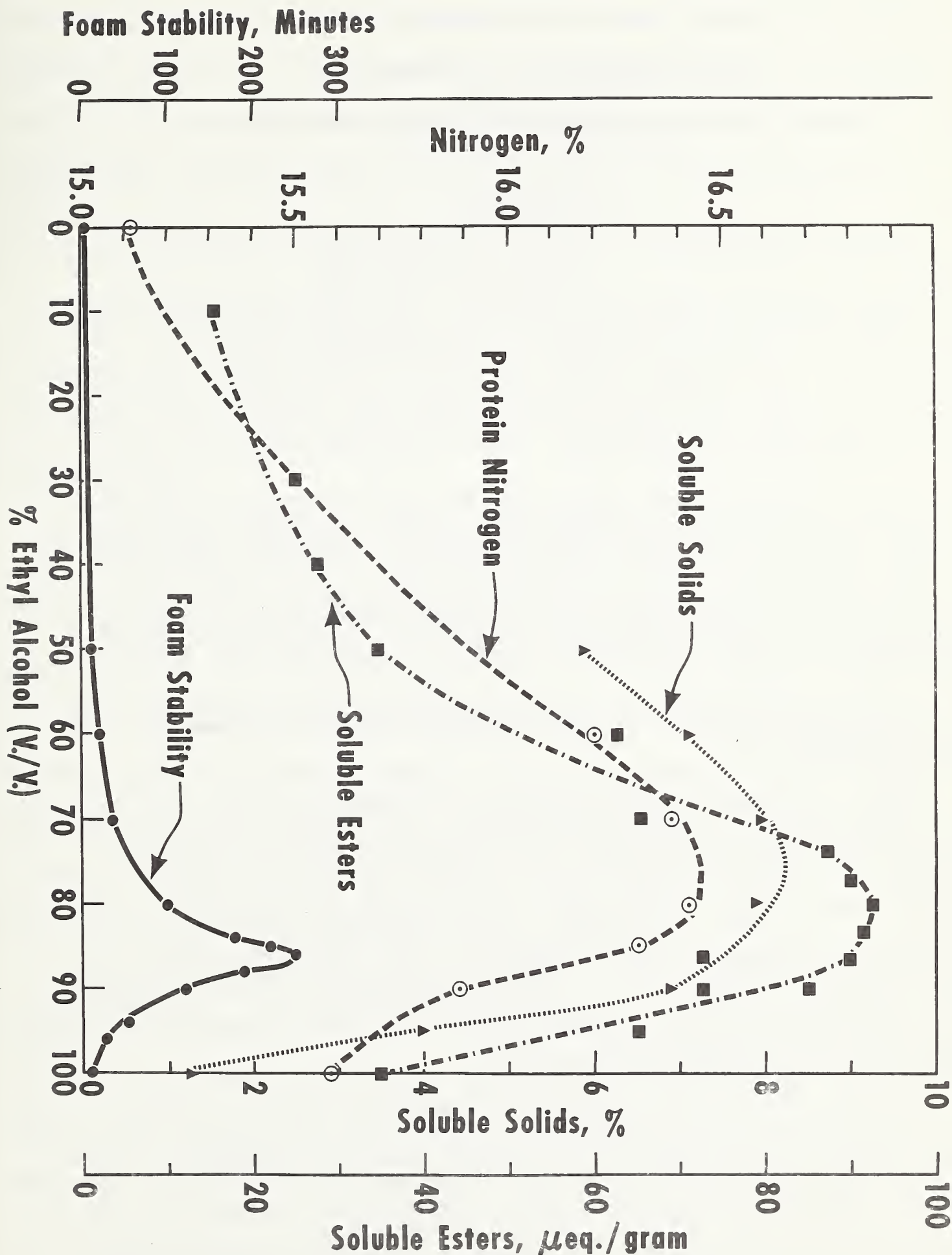


FIGURE 4

Effect of ethanol concentration on the amount of solids and esters removed from soybean globulins, and the nitrogen content and foam stability of soybean globulins after alcoholic extraction.

## SOME PROPERTIES OF PURIFIED EDESTIN

D. M. Stockwell, J. M. Dechary and

A. M. Altschul

Southern Utilization Research and Development Division  
New Orleans, Louisiana

### Introduction

Edestin from hempseed is easily crystallized and is therefore one of the most studied of the seed proteins. While there is general agreement that this protein as prepared is not monodisperse, there is no information on the chromatography of this protein on modified cellulose and its purification by such means. One of the difficulties with any method of purification which depends on the ionic strength of the medium is that edestin is much less soluble than most of the other seed proteins. For this reason chromatography at low ionic strength is difficult and electrophoresis must be performed in media of such high ionic strength as to render questionable the conclusions which might be drawn from that type of analysis.

There is, however, another property of edestin which may compensate for its low solubility and that is its relative stability to exposure to high temperatures. Indeed there is a pH range (5.8-7.8) within which no edestin is coagulated even by heating at 100° for twenty minutes (1). The solubility of edestin is far greater at 50° than at 25°; an advantage might accrue to chromatographing at 50°.

It turns out that the solubility of edestin is not an intrinsic property, but is the result of the various methods of preparation of the protein. One unusual outcome of these investigations is the effect of criminal law on the solubility of edestin. In certain countries such as

the United States it is not permitted to sell or have in possession viable hempseed. Hence all of the hempseed of commerce and presumably the source material for most crystalline edestin preparations is nonviable, heat-treated seed. Edestin prepared from such seeds is chromatographically different and less soluble than edestin prepared from viable seeds.

#### Materials and Methods

Seed. Fresh viable hempseeds were obtained through the cooperation of the Internal Revenue Service from their seizure number 4654. Edestin prepared from this viable sample was compared to that from commercially available seed.

Preparation of Edestin. Edestin was prepared by the classical principle of extracting at 50°C., and precipitating at 4°C. The final product was collected by centrifugation and dried rapidly over  $P_2O_5$  at room temperature.

Twice reprecipitated edestin prepared using a technique identical in all details is a consistent product. It is not separated from the sodium chloride from which solution it is finally precipitated; the dry product is brown, presumably due to the presence of some phenol oxidase. Kjeldahl nitrogen on a salt-free sample was 17.5%; sulphur was 1.07%.

Chromatography on DEAE cellulose at 50°C. Several buffer systems examined with respect to the solubility of edestin, such as borate and Tris buffers offered no advantage. Benzoate ion dissolved edestin quite readily, but benzoate is a poor buffer, and combinations of benzoate and phosphate in a buffer system gave uneven elutions during chromatography.



Therefore, the chromatography was conducted in phosphate buffer of pH 8, containing 15 micrograms per ml. of mycifradin sulfate, as an anti-bacterial agent.

Commercial DEAE cellulose was washed with 1 N sodium hydroxide, then with distilled water, and finally with ethanol and dried under vacuum at 40°C. About three grams of the dried cellulose were slurried in the buffer and deaerated by heating at 50°C. under vacuum for an hour. The slurry was immediately poured onto a 0.9 x 20 cm. column, water-jacketed at 50°C. and the cellulose packed under five p.s.i. pressure.

After washing with two liters of boiled deaerated buffer, delivered from beneath a layer of paraffin oil, the column was ready for use.

Solutions of protein were prepared by dialyzing the twice reprecipitated edestin against two liters of the same buffer for eighteen hours at 50°C. The solution was clarified by filtering at 50°C. through an ultrafine fritted filter under five pounds pressure.

Approximately 10 milligrams of protein were applied to the column at a concentration of 1 mg./ml. and the proteins were eluted from the column by linear gradient of 0 to 0.6 molar sodium chloride in buffer, unless otherwise noted. A syringe tip at the bottom of the column was adapted directly to a fine bore polyethylene tube, an arrangement which prevents loss of water by evaporation at the elevated temperature; the eluate was collected in 5 ml. fraction which were assayed for protein by the method of Lowry, et al. (2) and for chloride by the method of Asper, Schales, and Schales. (3)

Solubility. Solubility was measured by incubating measured amounts of solid in the solvent; after incubation for several hours at the

required temperature, the material was either passed through an ultrafine filter or centrifuged at 15,000 x g. The nitrogen content of the clear dissolved material was determined by the Kjeldahl method and multiplied by a constant factor to convert to protein solubility.

## Results

Chromatography of edestin at elevated temperatures. Figure I shows the chromatographic pattern of an extract of hempseed chromatographed at 50°C. immediately after extraction and filtering, and prior to any purification. It will be noted that the total hempseed protein may be divided into two groups: Those which do not adsorb onto the DEAE column at all and the group which adsorbs. This pattern resembles in general the pattern obtained for the total proteins of the peanut in that there too is a group I which did not adsorb on the column and there were three groups which could be chromatographed. In this respect the total proteins of the hempseed form a much simpler pattern than those of the peanut. In the preparation of edestin all of the soluble protein remains in solution so that crystalline edestin is practically free of the first group of proteins shown in Figure I.

The proteins in crystalline edestin can be fractionated further by chromatography at a higher protein-to-cellulose ratio and by successive elution at two temperatures. Twice reprecipitated edestin was put on a column (2.2 x 22 cm.) at 50°C. at a ratio of 400 mg. protein to 25 g. of DEAE cellulose. Then it was washed with the same buffer at 50°C. followed by 0.15 M NaCl in buffer at 25°C., and then with the same level of NaCl

at 50°C. Under these circumstances there is further fractionation of the "purified" edestin as is shown in Figure II. We have named the material which is eluted by salt at room remperature, Fraction II.

Fraction II was examined by ultracentrifugation and could not be distinguished from the original edestin by this criterion. The value of  $S^{\circ}_{20}$  for fraction II was 13.4 with a small amount of a faster sedimenting component with  $S^{\circ}_{20}$  of 20.3; compared to 13.7 and 19.9 for edestin . . . only difference is slightly greater solubility of II as indicated by peak heights.

The total ionic strength of the eluate at the point where protein fractions are eluted from DEAE cellulose is quite reproducible. Numerous studies on  $\alpha$ -conarachin (4) have yielded results within a range of 0.02 M NaCl concentration. It is interesting to note that protein extracts from commercial hempseed and commercial twice recrystallized edestin elute at significantly higher ionic strengths than do the preparations from unheated seed. It is also evident that Fraction II corresponds most closely to edestin and to the original major protein from hempseed extracts.

Properties of Fractions. Table I gives the composition of the various fractions as compared to the original edestin. Fraction I is apparently a nonprotein component, but yet gives a reaction with Lowry reagent. The role of Fraction I in the mixture we call edestin is interesting to speculate about, but as yet there is no information about the identity of this fraction.

Perhaps the major difference between the fractions lies in their solubility. At a ratio of 5 mg. solids per ml. buffer, over 2 mg. of Fraction II are dissolved per ml., compared to approximately 1.2 mg. of edestin prepared from viable seeds, and only 0.3 mg. of commercial edestin.



## Discussion

It is apparent from the data presented on edestin prepared from viable, unheated seed, that the solubility of the preparation is dependent on the previous history of the hempseed. From the work of Putzeys and Mahieu (1) it would seem reasonable to assume that heating protein solutions should not greatly influence the solubility of the protein, but heat applied to the seed (5) would be expected to promote chemical changes which would be reflected in the properties of the proteins. That this is so is illustrated by the low solubility of the commercial edestin as compared to the laboratory preparation, and also the more globulin-like nature of the commercial preparation when it is examined chromatographically in DEAE cellulose.

The chromatographic pattern of extracts of viable hempseed is remarkably simple in contrast to that obtained, for example, from the peanut. In the latter instance a quite complicated pattern of a group of globulins is obtained in addition to the unadsorbed group. The significance of the existence of a single protein group in a seed such as hemp may be a reflection of stronger interaction of the soluble proteins. As this work seems to show, at least three materials combine in the one protein band. Indeed, from an inspection of the solubility curves for edestin and its fractions it can be seen that even Fraction II does not exhibit the behavior of a single species of molecule in the solubility test and therefore it also must be considered to be a mixture.

The preparation of edestin used in this work is very similar to an ultracentrifugally monodisperse edestin fraction which Johnson and coworkers prepared by relatively simple means (6, 7); it can be estimated that at least 90% of the preparation used here had the same sedimentation constant as the monodisperse fraction mentioned above, but yet it yielded three



distinct fractions on chromatography on DEAE cellulose. Moreover, it was not possible to distinguish between the original edestin and the main chromatographic fraction by sedimentation in the ultracentrifuge. In this work the criteria which serve to distinguish edestin from its main fraction are their solubility differences, their amino acid compositions, which are distinctly different in glutamic acid and arginine content, and temperature and salt programmed chromatography.

### References

1. P. Putzeys and L. Mahiey, Nature 194 (1962), 773.
2. O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall. J. Biol. Chem. 265 (1951), 265.
3. S. P. Asper, Jr., O. Schales, and S. S. Schales. J. Biol. Chem. 168 (1947), 779.
4. J. M. Dechary, K. F. Talluto, and A. M. Altschul, Nature 190 (1961), 1125.
5. I. E. Liener in Processed Plant Protein Foodstuffs, A. M. Altschul, ed. Academic Press, New York (1958), 79.
6. B. P. Brand, D. A. I. Goring, and O. Johnson. Trans. Faraday Soc. 51 (1955), 872.
7. D. A. I. Goring and P. Johnson. Arch. Biochim. 56 (1955), 448.
8. N. J. Fairbairn. Chem. Ind. (1953), 86.
9. R. Montgomery. Biochim. Biophys. Acta 48 (1961), 591.
10. R. J. L. Allen. Biochem. J. 34 (1940), 858.

### Discussion

- Dr. Joseph J. Rackis: Difference in amino acid composition between edestin and fraction II may not be significant.
- Dr. Abraham Marcus: If fractions are put back together, will the solubility of the mixture be the same as the original edestin?
- Mr. Donald M. Stockwell: We have not done this.

Table 1.

FRACTIONATION OF EDESTIN ON DEAE CELLULOSE

<u>Material</u>	<u>Elution Scheme</u> <sup>1/</sup>		<u>Protein Recovered</u> <sup>2/</sup>	<u>N</u>	<u>P</u> <sup>4/</sup>	<u>Carbohydrate</u> <sup>3/</sup>
	<u>Chloride</u> <u>M</u>	<u>Temp.</u> <u>°C.</u>	<u>%</u>	<u>%</u>	<u>%</u>	<u>%</u>
Edestin	---	---	---	16.9	0.13	0.19*
Fraction I	---	50	11.5	0.09	0.15	1.3
Fraction II	0.15	25	51.4	16.1	0.08	0.3
Fraction III	0.15	50	14.4	14.5	0.09	0.9

<sup>1/</sup> Chromatography carried out in phosphate buffer, pH 7.8, ionic strength 0.1.

<sup>2/</sup> Measured by the method of Lowry et al.<sup>(2)</sup>

<sup>3/</sup> Expressed as mannose, and determined by the anthrone method<sup>(8)</sup> except \* which was determined by the phenol-sulfuric acid method.<sup>(9)</sup>

<sup>4/</sup> Measured by the method of Allen.<sup>(10)</sup>

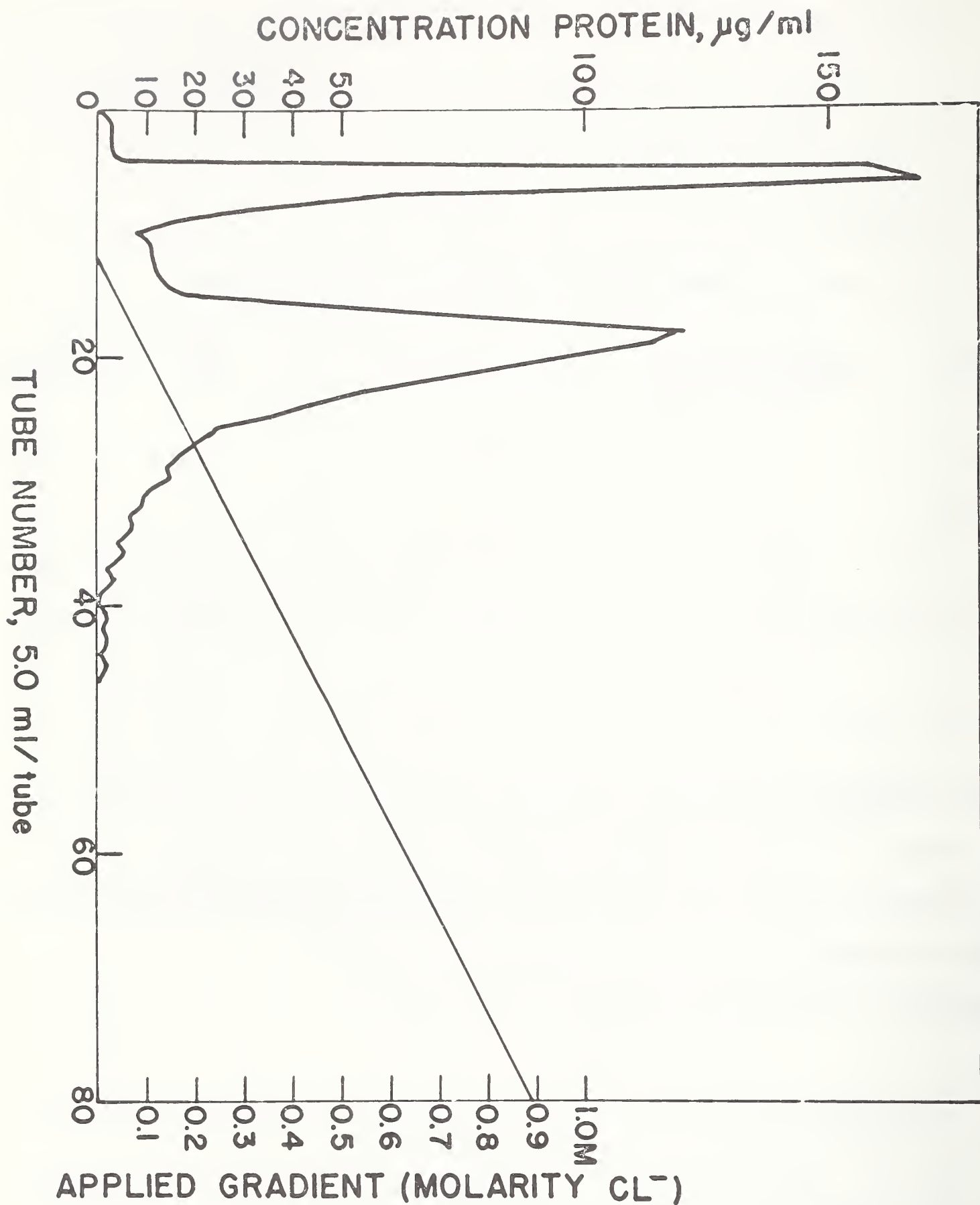


Figure I.

Fractionation of hempseed extract on DEAE cellulose at 50° C., using a linear NaCl elution scheme (right axis).

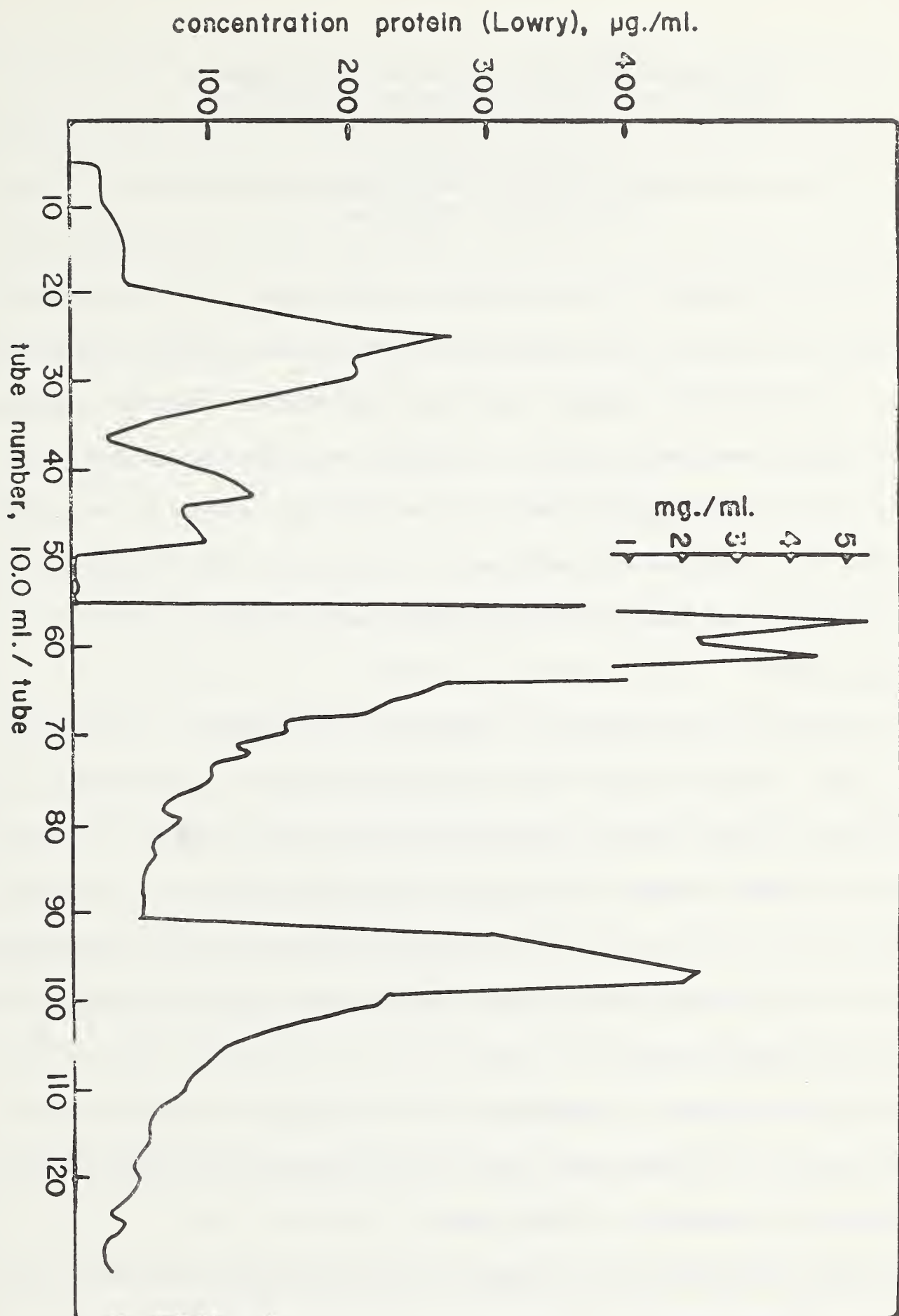


Figure II.

Fractionation of twice-reprecipitated edestin on DEAE cellulose; tubes 1-50 (Frac. I) eluted with phosphate buffer at 50° C., tubes 51-90 (II) eluted with 0.15 M  $\text{Cl}^-$  at 25° C., and tubes 91-130 (III) eluted with 0.15 M  $\text{Cl}^-$  at 50° C.



SOLUBLE PROTEINS OF THE PEANUT COTYLEDON;  
PURIFICATION AND PROPERTIES OF  $\alpha$ -CONARACHIN

Joseph M. Dechary  
Southern Utilization Research and Development Division  
New Orleans, Louisiana

In earlier studies (1) the proteins of the peanut were divided into two broad groups which were obtained by the ammonium sulfate fractionation of salt extracts of the peanut; these were selected as starting materials for chromatographic examination on chemically modified celluloses. The DEAE cellulose chromatograms showed the two fractions to be quite complex in nature, and a similar chromatogram of a phosphate buffer extract of the total protein of the peanut cotyledon demonstrated this even more strikingly (2).

Ultracentrifuge sedimentation patterns of the "40/85" or conarachin fraction were obtained at low ionic strength and high ionic strength. At 0.03 and pH 7.8 there were observed eight components, whereas at the same pH and in a buffer of ionic strength 0.2, there were only six. Changes in the sedimentation values which were observed and in the number of components indicated that there are one or more components of association-dissociation in this crude mixture (3,4).

The chromatographic properties of the more soluble fraction, the "40/85" fraction or conarachin, were determined inasmuch as these properties are affected by germination of the peanut.

The DEAE cellulose chromatograms of peanut cotyledon protein in the quiescent state and of three stages of germination of peanut cotyledons showed that one of the fractions of the conarachin group, Fraction III,

has been modified in some way so as to disappear from the chromatogram. In addition, the "O/40" or arachin group became more insoluble, as is indicated by the increase in salt concentration required to elute it.

The component, or fraction, named  $\alpha$ -conarachin which disappeared on germinating the peanut cotyledons was isolated from ungerminated peanuts by adsorption on TEAE and DEAE celluloses using NaCl gradient to elute the protein. The DEAE chromatogram of the isolated  $\alpha$ -conarachin indicated that the material is homogeneous in that the chromatographic technique can be a valid criterion of the homogeneity of a protein.

The sedimentation patterns of  $\alpha$ -conarachin in the ultracentrifuge in buffers of low and high ionic strength showed that in each instance there was one component with a trace of a second, lighter component. The sedimentation coefficients were 2 for the minor, light component and 13.1 for the major, heavy component in 0.03 ionic strength buffer, and 2 and 10.3 in buffer of 0.2 ionic strength. It would appear that we have isolated one of the association-dissociation systems mentioned earlier with reference to the conarachin group.

$\alpha$ -conarachin contains about 2% mannose which is firmly bound to it and which can be liberated by mild treatment with acid and detected by paper chromatography. The amino acid composition of the protein is that of a typical plant protein and the data fit rather well that of a protein having a minimum molecular weight of about 10,000. A molecular weight of 290,000 in the associated state is indicated by ultracentrifuge measurements made on the protein.

At the present time nothing is known about the role of  $\alpha$ -conarachin in the germination processes of the peanut. It is significant that its disappearance or modification takes place early, within four days of the inception of the germination process when the reserve proteins of the cotyledons are still at a high level.

#### References

1. Osborne, T. B., "The Vegetable Proteins", Longmans and Green Co., New York, 1924.
2. Dechary, J. M., Talluto, K. F., Evans, W. J., Carney, W. B., and Altschul, A. M., *Nature* 190: 1125 (1961).
3. Johnson, P. and Naismith, W. E. F., *Discussions Faraday Soc.*, No. 13: 98 (1953).
4. Evans, W. J., Carney, W. B., Dechary, J. M., and Altschul, A. M. *Arch. Biochem. Biophys.* 96: 233 (1962).

#### Discussion

Dr. Sidney J. Circle:

What about the lipid components? Nothing was mentioned about defatting.

Dr. Joseph M. Dechary:

Defatted peanut meal and whole peanuts give essentially the same chromatogram. In the case of the whole peanuts, the fat is associated with the arachin fraction and comes to the top upon centrifugation and is discarded.

\_\_\_\_\_:

The lysine content of  $\alpha$ -conarachin is high compared to that found in peanut meal.

## SEED HEMAGGLUTININS

Irvin E. Liener  
Department of Agricultural Biochemistry  
University of Minnesota  
St. Paul, Minnesota

### Introduction

That the seeds of certain plants are highly toxic to man and animals has been known for a long time. During the latter part of the 19th century when the science of bacteriology was becoming of age and having a marked influence on the scientific thinking of the times, it was generally believed that the toxicity of these seeds was due to bacterial toxins. The bacterial theory of seed toxins was disproved when, in 1884, Warden and Waddel (1) showed that the toxicity of the seed from the plant Abrus precatorius resided in a fraction which could be precipitated by alcohol from an aqueous extract of the seed and to which they gave the name abrin. Several years later Dixon (2) obtained a highly toxic concentrate from extracts of the castor bean, Ricinus communis. Stillmark (3), however, appears to have been the first to make the observation that an extract of the castor bean which he called ricin, as well as extracts from other toxic seeds, were capable of agglutinating the red blood cells of various animals. His work attracted the attention of Ehrlich who at the time was studying immunology at the Institute of Infectious Diseases in Berlin under Robert Koch. Ehrlich (4) demonstrated that guinea pigs and rats could be immunized against ricin by gradually increasing the level of the ingested seed. He also showed that anti-ricin serum was able to protect rats against toxic doses of ricin and likewise prevented the agglutination of red blood cells by ricin.



In 1907 Landsteiner and Raubitschek (5) showed for the first time that even edible species of such common legumes as the navy bean, lentil, and garden pea contained substances which could agglutinate the blood of various species of animals. Landsteiner (6) subsequently pointed out that seed proteins exhibit a certain degree of specificity regarding the species of animal from which the blood is derived. For instance, an extract from the lentil bean agglutinates the blood of rabbits quite readily but is without effect on pigeon blood, and conversely with a navy bean extract. This specificity of plant agglutinins toward certain species of animals has since been extended to cover a wide variety of plants and the blood of many species of animals (7,8).

Up until about 1948 the seed agglutinins remained nothing more than a laboratory curiosity of academic interest. In that year Renkonen (9) in Finland, and one year later, Boyd (10) in this country, reported that extracts of certain seeds exhibited a high degree of specificity towards human red cells of various blood groups. This discovery aroused the interest of immunologists and thus began an intensive systematic investigation of literally thousands of plants from all over the world with regard to their potential use as blood-typing reagents. The literature dealing with this part of the story has been covered in a number of excellent reviews (11-15) and should be consulted by those who are interested in this aspect of plant hemagglutinins.

Much of the immunological work dealing with the reaction of the seed agglutinins with red blood cells has involved the use of crude aqueous or saline extracts of the ground seed or, in some cases, the use of concentrates

partially purified by salt fractionation. The only seed hemagglutinins which have been purified to a sufficient extent to permit meaningful measurements of their chemical and physical properties are those from the castor bean (Ricinus communis), jack bean (Canavalia ensiformis), soybean (Glycine max), and beans belonging to the species Phaseolus vulgaris. This review then will deal primarily with the physical and chemical properties of the hemagglutinins derived from the seeds of these plants. In addition, studies relating to the possible nutritional significance of these substances will be described. Finally, experiments designed to elucidate the mechanism of interaction between red blood cells and seed agglutinins will be presented.

### Ricin

Because ricin was toxic when injected into animals in such extremely small amounts, there was once considerable skepticism as to whether ricin was really a protein. The observation that prolonged digestion with trypsin failed to destroy its toxicity (16) did little to support the belief that ricin was a protein. Highly toxic preparations of ricin were subsequently prepared by Osborne et al. (17) and Karrer et al. (18), and their work left little doubt that the toxicity of ricin was associated with its protein content. By means of fractionation with sodium sulfate, Kabat et al. (19) isolated a preparation of ricin which was immunochemically, electrophoretically, and ultracentrifugally homogeneous (see Table I for physical constants) but which had only two-thirds of the toxicity of a crystalline form of ricin isolated by Kunitz and McDonald (20). From this Kabat et al. concluded that their preparation of ricin was probably composed

of two agglutinins, only one of which was toxic however. In view of the uncertainty regarding the purity of ricin preparations which appear homogeneous by physical criteria, much of the work relating to the physical and chemical properties of ricin (21-25) must be accepted with some degree of reservation.

More recently, Funatsu (26) has been able to fractionate crystalline ricin on hydroxyl apatite into two components - one which was toxic but had no hemagglutinating activity, and the other which had strong hemagglutinating activity but was devoid of toxicity. The non-toxic hemagglutinin could be purified to the point where it was ultracentrifugally homogeneous by successive chromatographic runs on DEAE- and CM-cellulose (27). Its physical properties are recorded in Table I. The significance of this work lies in the fact that it represents the first time that it has been possible to demonstrate that the toxicity and hemagglutinating activity of a seed hemagglutinin are properties of two different species of protein.

#### Concanavalin A

Concanavalin A is the name given to one of the globular type proteins isolated in crystalline form from jack bean meal by Sumner (28). This protein not only agglutinated red blood cells but also has the unique capacity to precipitate glycogen (29, 30). Cifonelli et al. (31-33) have shown that concanavalin A reacts specifically with glycogen and certain mucopolysaccharides and bacterial polyglucosans but not with amylopectin. Although the physical properties of concanavalin A have been established (34) (see Table I), its chemical properties are largely unknown except for its content of cystine, tyrosine, and tryptophan determined before the advent



of modern techniques for amino acid analysis (34). This protein would appear to offer an excellent model for studies involving the interaction of protein and carbohydrate.

The direct injection of concanavalin A into animals causes agglutination of the red blood cells, followed by hemolysis, and ultimately death (36-38). The protein of the jack bean meal is of poor nutritive value unless heated (39) and has been reported to cause a variety of pathological lesions when fed to rats (40) or cattle (41). It remains to be proved, however, whether the harmful effects accompanying the ingestion of raw jack bean meal are actually due to concanavalin A.

#### Soybean hemagglutinin

Our own laboratory has, for some time, been interested in the explanation for the beneficial effect which heat exerts upon the nutritive value of the protein of the soybean (see review by Liener (42)). The trypsin inhibitor had been implicated as the most likely factor involved here, presumably by virtue of its ability to interfere with the normal digestive process in the intestinal tract. Somewhat disturbing however, was the observation that active antitryptic preparations retard the growth of animals even when incorporated into diets containing predigested protein (43, 44), suggesting that an inhibition of intestinal proteolysis was not the sole explanation for the poor nutritive value of raw soybean meal. In order to avoid intestinal proteolysis, it was decided to by-pass the gastrointestinal tract by injecting concentrates of the trypsin inhibitor directly into rats (45). As shown in Table II, injections of the crude trypsin inhibitor\*

---

\* The protein precipitated from the soybean whey with 0.7 saturated ammonium sulfate.



did in fact inhibit growth more or less in proportion to the frequency of injection, a lethal effect being produced by daily injections. When the crystalline inhibitor of Kunitz was injected at a level providing more than twice the level of the antitryptic activity of the crude preparation, the animals grew as well as the uninjected controls (Table III).

From these results, it became apparent that the toxicity of a crude preparation of trypsin inhibitor could not be explained by its antitryptic activity, but must be due to some other factor. Initially, progress in the purification of this toxic component was rather slow because the assay method for toxicity was time-consuming and required a large number of animals. It then occurred to us that we might be dealing here with a toxic protein similar to ricin which has hemagglutinating properties. Our toxic fractions were found to be very active hemagglutinating agents; the toxicity closely paralleling hemagglutinating activity. By following the hemagglutinating activity during the course of the purification of the toxic factor, we were able to isolate the toxic factor or soybean hemagglutinin (until shown otherwise these two properties are assumed to be associated with the same protein) in a high state of purity (46).

In order to assess the nutritional significance of the soybean hemagglutinin, it became necessary to prepare large amounts of the purified protein which could then be incorporated into diets fed to rats. When the purified hemagglutinin was added to diets at a level of activity equivalent to raw soybean meal, the growth inhibition which resulted was about one-half that produced by raw soybean meal itself (Table IV). From this we can conclude that the soybean hemagglutinin is at least partially responsible for the poor nutritive value of raw soybean meal, but the manner in which this substance exerts such an effect is still unknown.

A rather detailed study was made of the physical and chemical properties of the soybean hemagglutinin (48, 49). This protein appeared to be essentially homogeneous by several criteria: chromatography on DEAE-cellulose (Fig. 1), moving boundary electrophoresis at several pH values (Fig. 2), electrophoresis on a vertical starch column (Fig. 3), and ultracentrifugation (Fig. 4). The physical properties of the soybean hemagglutinin are summarized in Table I where they may be compared with other purified seed hemagglutinins. The amino acid composition of the soybean hemagglutinin was determined by ion-exchange chromatography with the results shown in Table V. There is nothing particularly unusual about the amino acid content of this protein except for the striking fact that there appears to be about 10% (by weight) glucosamine. The identity of this sugar was confirmed by a variety of chemical tests. End group analysis by Sanger's technique revealed the presence of two N-terminal alanine residues. Carboxypeptidase release serine and alanine from the C-terminal portion of the molecule. Evidently the soybean hemagglutinin molecule is comprised of at least two chains but the manner in which these two chains are held together is not known.

#### Hemagglutinins from Phaseolus

Partially purified concentrates of hemagglutinins from various species belonging to the genus *Phaseolus* had been prepared by a number of early investigators (50-52). More recent attempts to effect further purification of these hemagglutinins have produced divergent data with respect to the chemical and physical properties of these proteins. Rigas and Osgood (53) describe the purification of the hemagglutinin from the kidney bean (*Phaseolus vulgaris*) which they found to be a mucoprotein containing about

50% carbohydrate material. Hemagglutinating activity, however, was associated solely with the protein component which could be dissociated from the polysaccharide moiety under acid conditions. Using essentially the same purification procedure, Coulet et al. (54) obtained a protein whose properties were decidedly different from those described by Rigas and Osgood (see Table I). Prager and Speer (55) were able to show that a crude extract of Phaseolus vulgaris may be resolved into three hemagglutinating components by chromatography on DEAE-cellulose. Jaffé and Gaede (56) have likewise been able to obtain a number of hemagglutinating components by fractional precipitation with ammonium sulfate. The most active fraction precipitated between 55% and 70% saturation, and, because of its toxicity (see below), was called phaseolotoxin A. This fraction was essentially homogeneous by electrophoresis and ultracentrifugation having the properties shown in Table I. A less active fraction, "fraction B", obtained by fully saturating the supernatant with ammonium sulfate after separating phaseolotoxin A, consisted of three components, all of which had hemagglutinating activity but differed in physical properties from phaseolotoxin A. Jaffé is of the opinion that his fraction B is probably identical to the mucoprotein isolated by Rigas and Osgood since one of the components of this fraction contained about 30% carbohydrate which he identified as being composed of fucose, xylose, and galactose (57).

A divergence of opinion exists in the older literature regarding the toxicity of the hemagglutinins derived from Phaseolus (50-52, 58), which we may now attribute to the presence of more than one hemagglutinin of varying toxicity. Jaffé (59, 60) has made a thorough study of the toxicity of the various hemagglutinin fractions of Phaseolus vulgaris and found that they differed considerably in their toxicity when injected into mice with



no definite correlation between hemagglutinating activity and toxicity. Phaseolotoxin A was the most toxic of these fractions\*. With this fraction, however, partial denaturation by heat destroyed the hemagglutinating activity and toxicity to the same extent indicating that both activities are probably properties of the same protein.

Many reports exist in the literature concerning the toxic effects which may accompany the ingestion of raw legumes (see review by Liener (61)). Since the soybean hemagglutinin proved to be an effective growth inhibitor when fed to rats, we had reason to suspect that the hemagglutinins from Phaseolus might also exert a similar effect. Active hemagglutinating fractions isolated from the kidney bean and black bean were fed to rats at various levels in a basal diet containing 10% casein (62). As shown in Table VI, a definite inhibition of growth was observed at levels as low as 0.5%, although, in the case of the black bean, this level did not cause the death of any of the animals. Growth inhibition was much more marked at this level with the kidney bean hemagglutinin. In general, increasing the level of the hemagglutinin in the diet caused a greater inhibition of growth and shortened the period of survival, the effects of the kidney bean being much more severe in this respect than the black bean. Recently, we have shown that the kidney bean hemagglutinin will also inhibit the growth of chicks without causing pancreatic hypertrophy, a condition which is observed when raw kidney bean meal is provided in the diet (63).

Jaffé (59) has also reported that the growth of rats is markedly inhibited by phaseolotoxin A. Digestibility measurements (59, 64, 64a) revealed a sharp diminution in gross absorption as well as the digestibility

\* LD<sub>50</sub> of phaseolotoxin A was 50 mg/kg or approximately 1/700th and 1/40th as toxic as ricin and diphtheria toxin respectively.



of protein and fat. A 50% decrease in the absorption of glucose was observed in perfusion experiments with intestinal loops taken from rats fed this hemagglutinating fraction. Jaffé believes that the action of the hemagglutinin is to combine with the cells lining the intestinal wall (in much the same fashion as it combines with red blood cells), thus causing a non-specific interference of the intestinal absorption of all nutrients.

#### Mechanism of Hemagglutination

One of the interesting observations made with the seed agglutinins is the fact that their activity can be inhibited by a variety of simple sugars and oligosaccharides (65-69). In our laboratory we found that the activity of the hemagglutinin from the wax bean (Phaseolus vulgaris) could be inhibited by a variety of mucoproteins all of which contain sialic acid to a varying extent (see Table VII). This observation becomes meaningful in the light of what is known about the agglutination of red blood cells by certain viruses. This phenomenon has received considerable study (see review by Gottschalk (71)), and its salient features can best be summarized by reference to the schematic drawing shown in Fig. 5. A virus particle combines with so-called "receptor site" on the surface of the cell. This receptor site is believed to be composed of a type of mucoprotein of which one of the components is sialic acid. At 4°, combination of virus with the cell results in agglutination. If, however, the temperature is raised to 37°, the virus is eluted from the cell, and, at the same time, sialic acid is released from the receptor site. This is an enzymatic action in which an enzyme, presumably embedded in the virus and referred to as the "receptor destroying enzyme" (RDE) or neuraminidase, cleaves sialic acid from an adjacent sugar residue. Heat-inactivated virus proceeds only as

far as the agglutination stage; the enzymatic release of sialic acid does not take place. Red blood cells from which sialic acid has been removed can no longer be agglutinated by the virus. Virus can also act on certain mucoproteins (derived from such diverse sources as egg white, urine, meconium, cervix, bovine maxillary glands) in much the same way as with red blood cells, releasing sialic acid, the heat-inactivated virus again stopping at the absorption stage. If heat-inactivated virus is first treated with one of these mucoproteins, the virus is no longer capable of agglutinating red blood cells. Mucoproteins previously treated with unheated virus no longer inhibit viral agglutination. In other words, these mucoproteins inhibit viral agglutination by virtue of their structural similarity to the receptor site on the cell surface.

Returning now to the wax bean hemagglutinin, since the very same mucoproteins which inhibit viral agglutination also inhibit wax bean agglutination, it would appear that the wax bean hemagglutinin reacts with the same receptor site as does the virus. If this is true, then red blood cells which have been treated with virus and thus have had their receptor sites destroyed by the removal of sialic acid, should not be agglutinated by the wax bean hemagglutinin. Experimentally this was found to be true (70).

The receptor destroying activity or neuraminidase action of the virus can be duplicated by certain enzymes which have been isolated from such organisms as Vibrio cholero and certain species of Clostridia. These will cleave sialic acid either from the surface of red blood cells or from mucoproteins. RDE-treated cells can no longer be agglutinated, and RDE-treated mucoproteins are no longer inhibitory. An exactly analogous

situation was found to pertain to the wax bean hemagglutinin. When red blood cells were treated with RDE (from C1. perfringens), they were no longer agglutinated by the wax bean hemagglutinin, and when mucoproteins were treated with RDE they no longer inhibited the hemagglutinin. Thus all the evidence would seem to indicate that seed agglutinins combine with red blood cells at the same site as the virus, which, in effect, is a combination with a carbohydrate-containing moiety on the surface of the cell.

This affinity of seed agglutinins for carbohydrate-containing materials brings to mind the observation that concanavalin A, the hemagglutinin of the jack bean, precipitates certain polysaccharides. It may also be recalled that the soybean hemagglutinin was found to contain an appreciable amount of glucosamine and that some hemagglutinating fractions from Phaseolus vulgaris were quite high in carbohydrate content. These may represent some tightly bound carbohydrate material which was not completely eliminated during isolation. The affinity of hemagglutinins for carbohydrates may offer a possible explanation for their function in plants. According to Ensgraber (72), they may function as "carbohydrate fixers" and thus enable the plant to store carbohydrates in the seed. Since hemagglutinins initially appear in the leaves of young plants and then reappear in the seed as the plant matures, Susplugas and Coulet (73) suggest that they act as a means of translocating carbohydrate material in the growing plant. Regardless of their supposed function in the plant itself, the unique properties exhibited by these seed hemagglutinins in the test-tube and in the animal organism will no doubt continue to intrigue workers in the fields of immunology, nutrition, and biochemistry for many years to come.



## References

1. Warden, C. J. H. and Waddell, L. A., Calcutta, Bengal Secretarial Press, 1884, 76 pp.
2. Dixon, T., Australasian Medical Gazette 6, 155, 1886-7
3. Stillmark, H., Inaug. Diss. Dorpat, E. J. Karow, 1888.  
Cited by Osborne, T. B., The Vegetable Proteins, Longmans, Green and Co., New York, 1909.
4. Ehrlich, P., Deutsch. Med. Wochenschr. 17, 976, 1891.
5. Landsteiner, K. and Raubitschek, H., Centralbe Bakt. 45, 660, 1907.
6. Landsteiner, K., The Specificity of Serological Reactions, Charles C. Thomas, Springfield, Ill. 1936, p. 6.
7. Bird, G. W. G., Nature 172, 401, 1953.
8. Bird, G. W. G., Brit. J. Exp. Path. 35, 252, 1954.
9. Renkonen, K. O., Ann. Med. Exper. Biol. Fenn. 26, 66, 1948.
10. Boyd, W. C. and Reguera, R. M., J. Immunol. 62, 333, 1949.
11. Bird, G. W. G., Acta Chir. Belgica, Suppl. 1, 33, 1954.
12. Eyquem, A., Saint-Paul, M., and Magnin, J., Ann. de l' institut Pasteur 89, 60, 1955.
13. Krupe, M., Blood group-specific plant proteins (phyto agglutinins), Ferdinand Enke Verlag. Stuttgart, Germany, 1956.
14. Bird, G. W. G., Brit. Med. Bull. 15, 165, 1959.
15. Saint-Paul, M. Transfusion 4, 3, 1961.
16. Jacoby, M., Physiol. u. Pathol. 1, 51, 1902.
17. Osborne, T. B., Mendel, L. B., and Harris, I. F., Am. J. Physiol. 14, 259, 1905.
18. Karrer, P., Smirnoff, A. P., Ehrensperger, H. Van Slooten, I., and Keller, M., Z. Physiol. Chem., 135, 129, 1924.
19. Kabat, E. A., Heidelberger, M., and Bezer, A. E., J. Biol. Chem. 168, 629, 1947.
20. Kunitz, M. and McDonald, M. R., J. Gen. Physiol. 32, 25, 1948.
21. Levy, M. and Benaglia, A. E., J. Biol. Chem., 186, 829, 1950.
22. Moule, Y., Bull. Soc. Chim. biol. 33, 1467, 1951.
23. Moule, Y., Arch. sci. physiol. 5, 227, 1951.
24. Furihata, R., J. Biochem. (Japan) 38, 361, 1951.
25. Morgue, M., Grebus, C., Baret, R., and Kassata, R., Comp. Rend. soc. biol. 154, 1845, 1960.
26. Funatsu, G., J. Agr. Chem. Soc. Japan, 34, 139, 1960
27. Takahashi, T., Funatsu, G., and Funatsu, M., J. Biochem. (Japan) 51, 288, 1962: 52, 50, 1962.
28. Sumner, J. B., J. Biol. Chem. 37, 137, 1919.
29. Sumner, J. B. and Howell, S. F., J. Immunol. 29, 133, 1935.
30. Sumner, J. B. and Howell, S. F., J. Bacteriol. 32, 227, 1936.
31. Cifonelli, J. A., Montgomery, R. and Smith, F., J. Am. Chem. Soc. 78, 2485, 1956.
32. Cifonelli, J. A., Montgomery, R. and Smith, F., J. Am. Chem. Soc. 78, 2488, 1956.
33. Cifonelli, J. A. and Smith, F., J. Am. Chem. Soc. 79, 5055, 1957.
34. Sumner, J. B., Gralen, N., and Eriksson-Quensel, L.-B., J. Biol. Chem. 125, 45, 1938.



35. Sumner, J. B. and Graham, V. A., J. Biol. Chem. 64, 257, 1925.
36. Ham, T. H. and Castle, W. B., Trans. Assoc. Amer. Phys. 55, 127, 1940.
37. Dameshak, W. and Miller, E. B., Arch. Int. Med. 72, 1, 1943.
38. Lee, J. S., Puh, C., and Tsai, C., Proc. Chinese Physiol. Soc., Chengtu Branch 2, 59, 1944.
39. Borchers, R. and Ackerson, C. W., J. Nutrition 41, 339, 1950.
40. Orru, A. and Demel, V. C., Quademi Nutriz. 7, 273, 1941.
41. Shone, D. K., Rhodesia Agric. J. 58, 18, 1961.
42. Liener, I. E., In: Processed Plant Protein Foodstuffs, Chapt. 5 Edited by A. M. Altschul, Academic Press, New York, 1958.
43. Liener, I. E., Deuel, H. J. Jr., and Fevold, H. L., J. Nutrition 39, 325, 1949.
44. Desikachar, H. S. R. and De, S. S., Science 106, 421, 1947.
45. Liener, I. E., J. Biol. Chem. 193, 183, 1951.
46. Liener, I. E. and Pallansch, M. J., J. Biol. Chem. 197, 29, 1952.
47. Liener, I. E., J. Nutrition 49, 527, 1953.
48. Pallansch, M. J. and Liener, I. E., Arch. Biochem. Biophys. 45 366, 1953.
49. Wada, S., Pallansch, M. J., and Liener, I. E., J. Biol. Chem. 233, 395, 1958.
50. Weinhaus, O., Biochem. Z. 18, 228, 1908.
- 50a. Schneider, E. C., J. Biol. Chem. 11, 47, 1911.
51. Luning, O. and Bartels, W., Z. Untersuch. Lebensm. 51, 220, 1926.
52. Goddard, V. R. and Mendel, L. B., J. Biol. Chem. 82, 447, 1929.
53. Rigas, D. A. and Osgood, E. E., J. Biol. Chem. 212, 607, 1955.
54. Carlet, M., Bezou, M. J., and Cognet, B., Bull. soc. chim. Biol. 41, 1385, 1959.
55. Prager, M. D. and Speer, R. J., Proc. Soc. Exptl. Biol. Med. 100, 68, 1959.
56. Jaffé, W. G. and Gaede, K., Nature 183, 1329, 1959.
57. Jaffé, W. G., Acta Cientifica Venezolana 13, 100, 1962.
58. Piekarski, L., Dissertationes Pharmaceutical 9, 255, 1957.
59. Jaffé, W. G., Arzneimittelforschung 12, 1012, 1961.
60. Jaffé, W. G., Experientia 18, 76, 1962.
61. Liener, I. E., Am. J. Clin. Nutrition 11, 281, 1962.
62. Honavar, P. M., Shih, C.V., and Liener, I. E., J. Nutrition 77, 109, 1962.
63. Wagh, P. V., Klaustermeier, D. F., Waibel, P. E., and Liener, I. E., J. Nutrition, in press.
64. Jaffé, W. G. and Canejo, G., Acta Cientifica Venezolana 12, 59, 1961.
- 64a. Jaffé, W. G., Planchat, A., Praz Pumar, J. I., Torrealba, R. and Nully Franchesi, D., Arch. Venezolana Nutric. 6, 195, 1955.
65. Morgan, W. T. J. and Watkins, W. M., Brit. J. Exptl. Pathol. 34, 94, 1953.
66. Mäkelä, O., Ann. Med. exp. Fenn 35, Supplement 11, 1957.
67. Mäkelä, O., Nature, 184, 111, 1959.
68. Mäkelä, O., Mäkelä, P. and Lektovaara, R., Ann. Med. exp. Fenn. 37, 328, 1959.
69. Krüpe, M., Hoppe-Seyler's Z. physiol, chem. 299, 277, 1955.

70. Northrop, R. L. and Liener, I. E., Proc. Soc. Exptl. Biol. Med. 100, 105, 1959.
71. Gottschalk, R., Yale, J. Biol. Med. 28, 525, 1956.
72. Ensgraber, A., Botan. Gesell. 71, 8, 1958.
73. Susplugas, J. and Coulet, M., Soc. Pharm. Montpellier, April, 1954.

Table I

## Summary of the Physical Constants of Purified Seed Agglutinins

Plant		Name of protein	Sedimentation constant (S <sub>20</sub> )	Diffusion constant (D <sub>20</sub> )	Molecular weight	Isoelectric point	Reference
Botanical name	Common name						
<u>Ricinus communis</u>	Castor bean	Ricin	4.8	6.0	85,000	5.4-5.5	19
			3.9		36,000		20
<u>Canavalia ensiformis</u>	Jack bean	Concan- avalin A	6.39 6.0	5.6	98,000 <sup>a</sup> 96,000	5.5	27 34
<u>Glycine max</u>	Soybean		6.4	5.7	105,000	6.1	48,49
<u>Phaseolus vulgaris</u>	Kidney bean		7.2		89,000 <sup>a</sup>	6.5 <sup>b</sup>	53
			6.15			5.0-5.1	54
	Black bean	Phaseolo- toxin A	5.9		126,000	4-5	56,57

<sup>a</sup>Based on light-scattering measurements. All other molecular weight estimates based on ultracentrifugal and diffusion data.

<sup>b</sup>The mucoprotein from which this protein was derived had an isoelectric point of 5.6.

Table II.

Growth inhibitory effect of repeated injections of a crude trypsin inhibitor preparation. Taken from Liener (45).

Injection schedule <sup>a</sup>	Duration of experiment Days	Gain in weight gm/day
None	30	1.33
Once every 7 days	30	0.98
Once every 3 days	30	0.84
Once every 2 days	30	0.78
Daily	13-30 <sup>b</sup>	0.21

<sup>a</sup>Each dose was 130 mg per kg. body. Six animals per group.

<sup>b</sup>One animal died on the 13th day, one on the 16th, two on the 17th, and a fifth on the 20th day. The sixth animal survived the experiment.



Table III

Comparison of crude and crystalline trypsin inhibitor preparations when injected daily. Taken from Liener (45).

	Crude	Crystalline	Uninjected control
Level injected, mg/kg/day	130	50	0
Antitryptic activity injected units $\times 10^{-3}$ /kg/day	208	505	0
Duration of experiment	5-25 <sup>a</sup>	25	25
Gain in weight, gm/day	0.30	1.65	1.61

<sup>a</sup> One animal died on each of the following days: 5,13,20,21,22, and 25.

Table IV

Growth inhibitory effect of the soybean hemagglutinin (SBH)

Taken from Liener (47).

Protein component of diet	Wgt. gain <sup>a</sup> gm	% growth inhibition
25% heated soybean meal	60.0	0
25% raw soybean meal	28.0	53.2
25% heated soybean meal + 0.8% SBH	45.0	25.6

<sup>a</sup> Average of 4 animals over a period of 2 weeks.

Table V.

Amino acid composition of soybean hemagglutinin.

Taken from Wada et al. (49).

Amino acid	Percent by weight	Number of residues per mole (M.W. = 96.000)
Aspartic acid	14.7	106
Threonine	5.6	45
Serine	4.8	44
Glutamic acid	7.8	51
Proline	4.1	35
Glycine	3.1	40
Alanine	5.4	57
Valine	5.3	43
Methionine	1.3	8
Isoleucine	6.2	45
Leucine	3.5	26
Tyrosine	2.8	15
Phenylalanine	5.7	33
Histidine	2.2	14
Lysine	3.2	21
Ammonia	0.5	28
Arginine	5.7	32
Tryptophan	4.7	22
Cystine	1.5	6
Glucosamine	10.2	55
Total	98.3	726

Table VI

Effect of purified hemagglutinin fractions from the black bean  
and kidney bean on the growth of rats.  
Taken from Honavar et al. (62).

Source of hemagglutinin	Purified hemagglutinin in diet	Av. gain in weight	Mortality <sup>1</sup>
	%	gm/day	days
Black bean	0	+2.51	
	0.5	+1.04	
	0.5 <sup>2</sup>	+2.37	
	0.75	+0.20	
	1.2	-0.91	15-19
	2.3	-1.61	12-17
	4.6	-1.72	5-7
Kidney bean	0	+2.31	
	0.5	-0.60	13-16
	0.5 <sup>2</sup>	+2.29	
	1.0	-0.87	11-13
	1.5	-1.22	4-7

<sup>1</sup>100% mortality observed during period recorded here. Blank space indicates that no deaths were observed.

<sup>2</sup>Solution of hemagglutinin boiled for 30 minutes and dried coagulum fed at the level indicated. Hemagglutinating activity was completely destroyed by this treatment.



Table VII

Substances showing inhibition of agglutination by the wax bean hemagglutinin. Taken from Northrop and Liener (70).

Substance tested	Inhibiting activity	Sialic acid
	I.U./mg.	%
Ovomucin	520	5.8
Ovomucoid	10	2.0
Serum viral inhibitor	350	16.1
Rabbit serum	475 <sup>a</sup>	12.2
Bovine serum	16 <sup>a</sup>	16.0
Meconium	100	26.0
Sialyl-lactose	300	4.5

<sup>a</sup> Inhibitor activity and sialic acid expressed as inhibitor units (I.U.) per ml. and mg. % respectively.

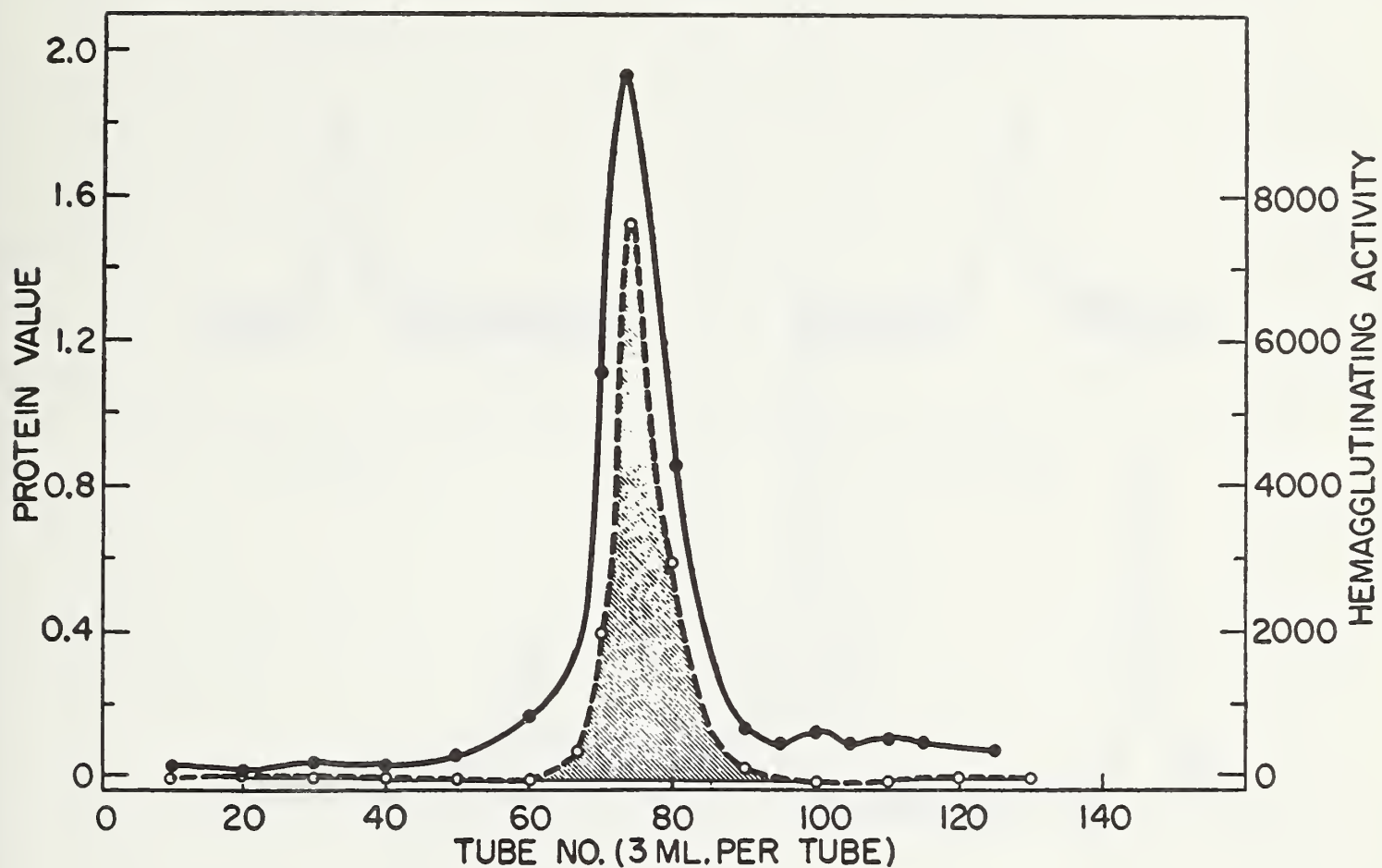


FIGURE 1

Chromatographic behavior of soybean hemagglutinin on DEAE-cellulose using gradient elution from 0.005 M borate buffer, pH 8.7, to 0.1 M  $\text{Na}_2\text{CO}_3$ , pH 9.

Absorbance at 280 mμ shown by solid line, and activity by shaded portion of curve. Taken from Wada et al. (49).

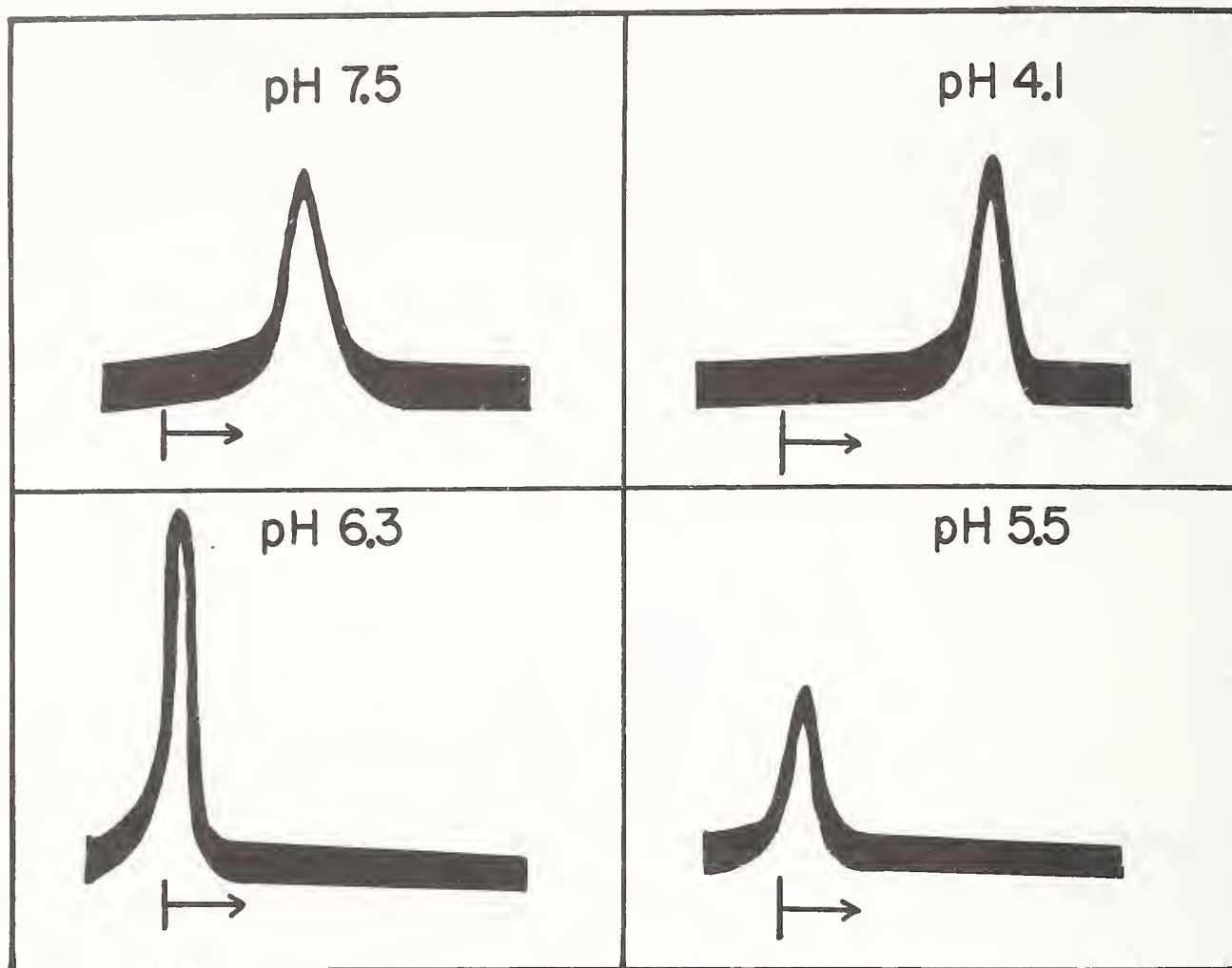


FIGURE 2

Electrophoretic patterns of soybean hemagglutinin at various pH values. At pH's 4.1 and 5.5, migration of the boundary is toward the cathode, and at pH's 6.3 and 6.7 migration is toward the cathode, and at pH's 6.3 and 7.5 migration is toward the anode. Taken from Pallansch and Liener (48).

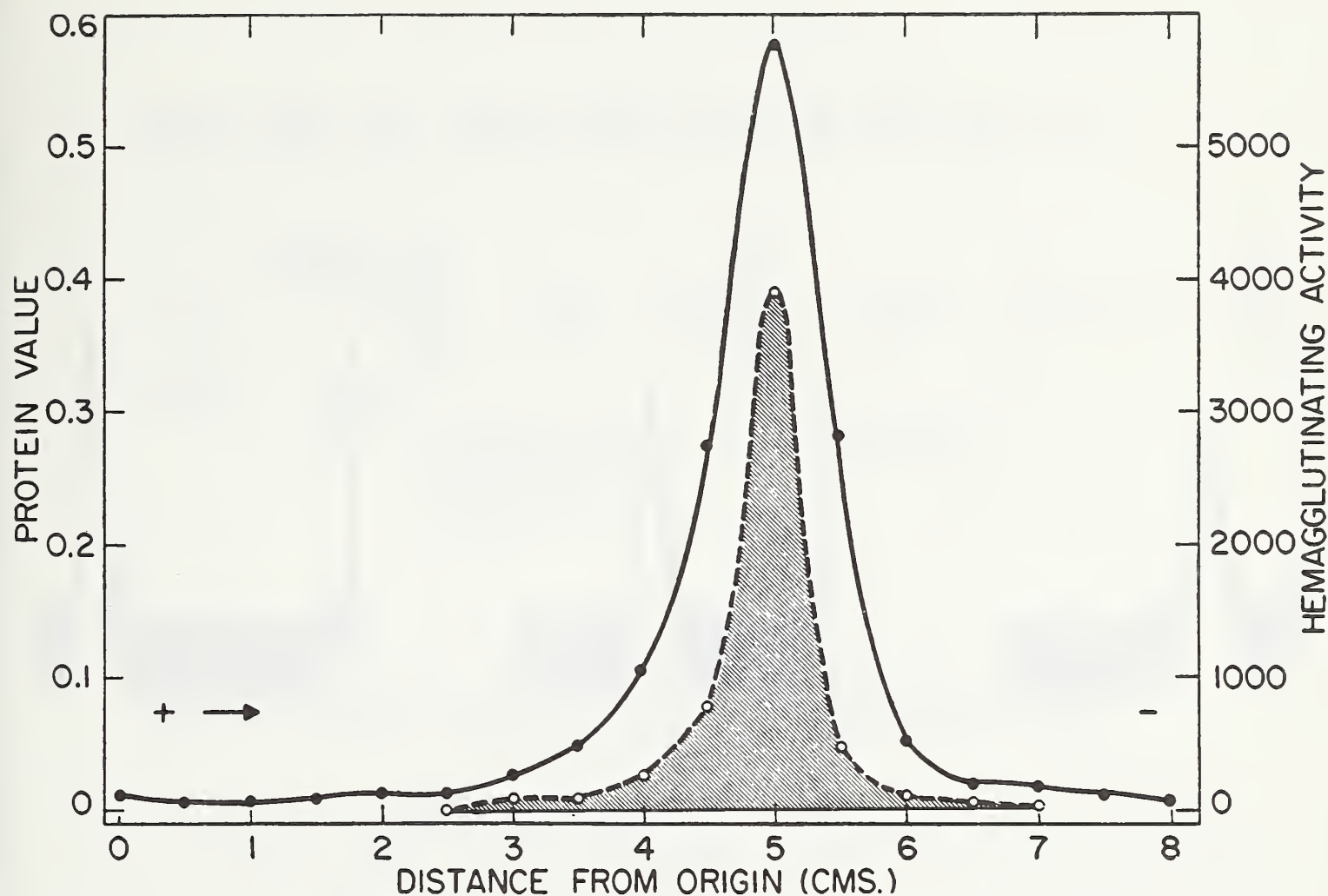


FIGURE 3

Electrophoretic behavior of soybean hemagglutinin on starch in 0.1 M acetate buffer, pH 4. Absorbance at 280 mμ shown by solid curve and hemagglutinating activity by shaded portion of curve. Taken from Wada et al. (49).

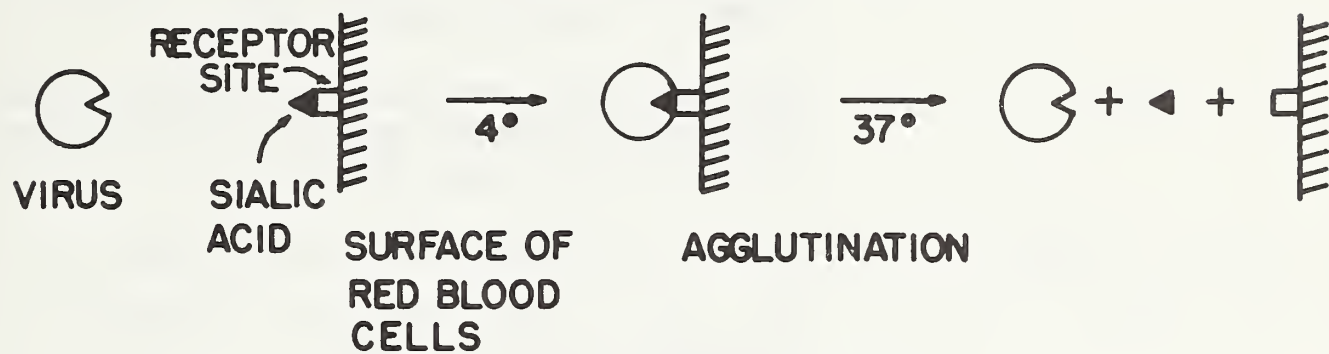




FIGURE 4

The sedimentation pattern of 1.5% solution of soybean hemagglutinin in 0.2 M NaCl as a function of time. Photographs from left to right were taken at intervals of 32 minutes. Taken from Pallansch and Liener (48).

## REACTION OF VIRUS WITH RED BLOOD CELLS



## REACTION OF VIRUS WITH MUCOPROTEINS

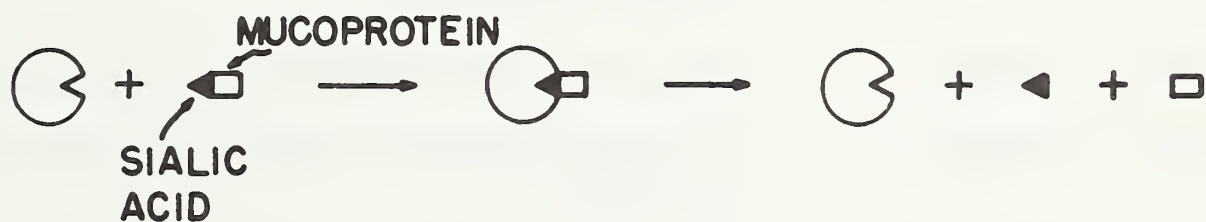


FIGURE 5

Schematic drawing showing the reaction of virus with red blood cells and mucoproteins.

## Discussion

- \_\_\_\_\_ :
- Dr. M. L. Anson: In lima beans, the hemagglutinin disappears in germinating seeds. These may be representative of reserve proteins; however, there is reason to doubt this.
- Dr. Irvin E. Liener: Why is it that the nutritive value of the raw soybean can be improved by the addition of one or two amino acids?
- Dr. M. L. Anson: Not all investigators believe this.
- Dr. M. L. Anson: Why aren't these inhibitory proteins digested in the stomach?
- Dr. Nathan Sharon: One of the soybean inhibitors is resistant to pepsin and acid treatment. (See Birk, Biochem. Biophys. 54 [1961] 378)
- Dr. Irvin E. Liener: Ricin from castor bean is resistant to trypsin hydrolysis.
- Dr. Joseph J. Rackis: Some of these inhibitors are heat stable.
- Dr. Mark A. Stahmann: Is hemagglutination affected by the ionic strength?
- Dr. Irvin E. Liener: This has not been studied, but agents such as urea inactivate hemagglutinins.
- Dr. Mark A. Stahmann: Poly-lysine will act as a hemagglutinating agent, and its action is affected by the ionic strength.
- Dr. Pierre Grabar: Some of the components participating in the hemagglutination become soluble in water after lyophilization of erythrocyte stroma.
- Dr. Nathan Sharon: It seems worthwhile to point out that the soybean hemagglutinin is the only plant protein shown to contain in its molecule an amino sugar (glucosamine, in this case). Furthermore, this is one of the few cases in which glucosamine was found in plants. I would now like to ask Dr. Liener if he is willing to comment on the chemical basis of toxicity?

Dr. Irvin E. Liener:

There may be a combination with the cells  
in vivo. Further study is necessary.



# ISOLATION AND CHARACTERIZATION OF SOYBEAN TRYPSIN INHIBITORS<sup>1</sup>

J. J. Rackis

Northern Regional Research Laboratory,<sup>2</sup> Peoria, Illinois

In 1946, Kunitz (1) isolated a crystalline trypsin inhibitor from acid extracts of raw soybean meal. Growth inhibition and pancreatic hypertrophy in nonruminants fed raw soybean meal have been attributed to the physiological action of water-soluble proteins having antitryptic activity (2). In all these studies, the trypsin inhibitor, prepared by a combination of water and salt extraction of raw meal, was of unknown purity. Now, two highly purified trypsin inhibitors have been isolated by chromatography on DEAE-cellulose.

Dehulled soybean meal, hexane-extracted, contains at least 50% protein ( $N \times 6.25$ ). Nearly 95% of the total nitrogen is in the form of protein nitrogen. Solubility of the nitrogen in raw soybean meal as a function of pH (3) is shown in Figure 1. Similar nitrogen solubility curves have been obtained with linseed (4), sunflower (5), cottonseed (6), and peanut (6) meals. These studies show that a large amount of protein in oilseed meals is soluble in either water or dilute alkaline solution and that a major portion of this soluble protein precipitates with acid in the pH range of 4.0-4.5.

---

<sup>1</sup> To be presented at Seed Protein Conference, New Orleans, La., January 21-23, 1963.

<sup>2</sup> This is a laboratory of the Northern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture.

In our laboratory, this solubility behavior is the basis for conducting research on the physical, chemical, and biological properties of soybean meal because fractionation of the meal can be readily achieved as outlined in Figure 2. The three advantages to this procedure are: (a) Planning research according to protein fractions is facilitated; (b) enzymes, trypsin inhibitors, and other physiologically active proteins are conveniently concentrated both in the water-insoluble residue and the water-soluble whey protein, and (c) the same procedure is followed industrially to produce a variety of soybean protein products for food, feed, and industrial use.

Presumably, a similar fractionation scheme might also prove advantageous in research on other oilseed meals because solubility characteristics and many physical and chemical properties, particularly those of the acid-precipitated proteins, resemble those in soybean meal.

The whey protein fraction contains most of the physiologically active proteins that inhibit growth and cause pancreatic hypertrophy in non-ruminants (7). Previous electrophoretic studies on soybean whey proteins have disclosed five distinct components. Two resolvable peaks appear in ultracentrifuge patterns, corresponding to components with  $S_{20w}$  values of 2S and 6S.

Initial chromatographic studies with DEAE-cellulose indicated that the whey protein fraction is a complex protein system composed of at least 13 distinct peaks (8), and as many as 20 peaks were resolved by use of a variable sodium chloride gradient (unpublished data). Paper electrophoretic techniques were of little value in resolving the proteins because most of them were irreversibly bound to the paper.

Preliminary studies with gel electrophoresis indicated only fair resolution of trypsin inhibitor activity. Nearly 99% of the trypsin inhibitor activity applied to the column was resolved into peaks corresponding to components K and M by gradient elution chromatography on DEAE-cellulose (Figure 3); these respective components are referred to as soybean trypsin inhibitor A<sub>1</sub> (SBTIA<sub>1</sub>) and A<sub>2</sub> (SBTIA<sub>2</sub>).

In chromatographic columns under conditions of near 100% adsorption (0.01M potassium phosphate buffer, pH 7.6, and 5-30 mg. of protein), SBTIA<sub>1</sub> and A<sub>2</sub> will have an RF of either 0 or 1, depending upon the sodium chloride concentration in phosphate buffer of the eluant. The resin column used in the development of the gradient elution diagram of Figure 3 has a holdup volume of about 25 ml. This indicates that the two trypsin inhibitor peaks, which were eluted from the column at volumes of 150 and 195 ml., respectively, began to move down the column with an RF=1 at eluant volumes of 125 and 170 ml. The sodium chloride concentration of the eluants at these points corresponds to approximately 0.16 and 0.21M sodium chloride. These are then the concentrations required to elute SBTIA<sub>1</sub> and A<sub>2</sub> in that order. Components A through J were arbitrarily divided into four areas. These divisions were used to separate whey protein into six fractions by stepwise elution with sodium chloride as indicated in Figure 4. Nearly 99% of the trypsin inhibitor activity was recovered in fractions V and VI, designated as SBTIA<sub>1</sub> and A<sub>2</sub>.

By discarding part of the protein in the leading edge of fraction VI, SBTIA<sub>2</sub> was found to be chromatographically pure and also homogeneous in electrophoresis and in ultracentrifugation.

SBTIA<sub>1</sub> required additional purification. This was accomplished by rechromatography on DEAE-cellulose preparative columns by using a continuous variable gradient device (9).

A comparison of some of the physical properties and antitrypsin activity of SBTIA<sub>1</sub>, SBTIA<sub>2</sub>, and Kunitz' crystalline soybean trypsin inhibitor, designated SBTI(5X), (10) is given in Table 1.



## References

1. M. Kunitz. J. Gen. Physiol 29, 149 (1946).
2. I. E. Liener in "Processed Plant Protein Foodstuffs," A. M. Altschul, ed., Chap. 5., Academic Press, New York (1958).
3. A. K. Smith and S. J. Circle. Ind. Eng. Chem. 30, 1414 (1938).
4. A. K. Smith, V. J. Johnsen, and A. C. Beckel. Ind. Eng. Chem. 38, 353 (1946).
5. A. K. Smith and V. J. Johnsen. Cereal Chem. 25, 399 (1948).
6. T. D. Fontaine, W. A. Pons, Jr., and G. W. Irving, Jr. J. Biol. Chem. 164, 487 (1946).
7. J. J. Rackis. Proc. Conf. on Soybean Products for Protein in Human Foods, Peoria, Illinois, September 1961, Agr. Res. Serv., U.S. Dept. Agr., ARS-71-22, p. 110 (1962).
8. J. J. Rackis, H. A. Sasame, R. L. Anderson, and A. K. Smith. J. Am. Chem. Soc. 81, 6265 (1959).
9. E. A. Peterson and H. A. Sober. Anal. Chem. 31, 857 (1959).
10. J. J. Rackis, H. A. Sasame, R. K. Mann, R. L. Anderson, and A. K. Smith. Arch. Biochem. Biophys. 98, 471 (1962).

Table 1.--Physical Properties and Antitrypsin Activity of Purified Soybean Trypsin Inhibitors

Property	SBTIA <sub>1</sub>	SBTIA <sub>2</sub>	SBTI (5X)
Sedimentation constant, S <sub>20w</sub>	1.80S	2.29S	2.30S
Partial specific volume, ml./g.	0.736	0.735	0.745
Molecular weight, g. mole <sup>-1</sup>	14,300	21,600	22,700
Electrophoretic mobility value, sq. cm./v./sec. <sup>1/</sup>	-7.4 X 10 <sup>-5</sup>	-8.0 X 10 <sup>-5</sup>	-8.0 X 10 <sup>-5</sup>
Extinction coefficient (E), 1 mg./ml. at 280 mμ, pH 7.6	0.942	0.994	0.900
Specific activity, μg. trypsin inhibited/μg. inhibitor <sup>1/</sup>	1.60	1.05	1.0
Nitrogen content, % <sup>2/</sup>	14.96	15.68	16.50

<sup>1/</sup> Average of five determinations.

<sup>2/</sup> On a moisture-free and ash-free basis.

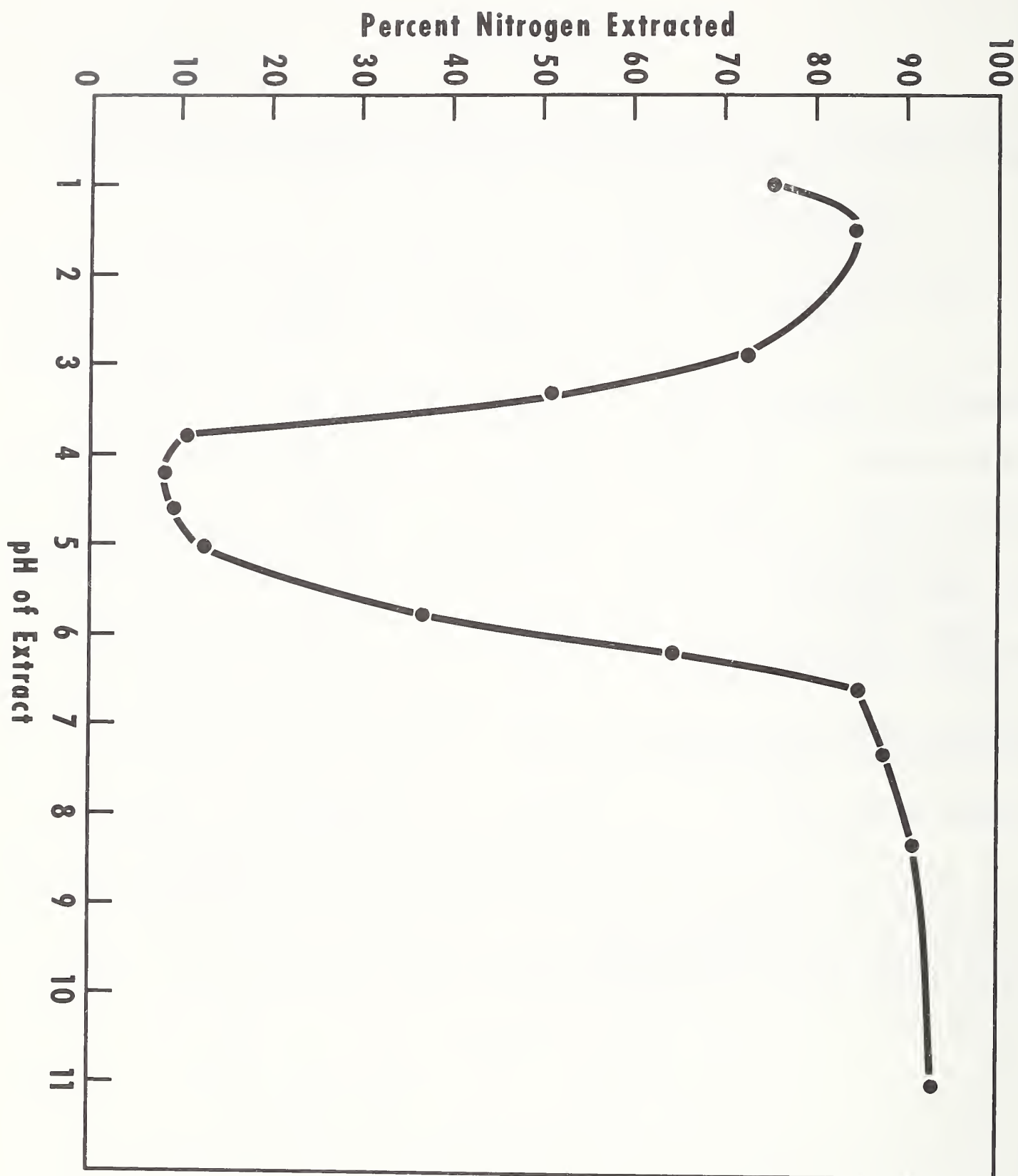


FIGURE 1  
Percentage of the total nitrogen of defatted soybean  
meal extracted at different pH values.

## Dehulled-Defatted Soybean Meal

Double extraction with  
water to meal ratio,  
10:1 and 5:1

Residue Protein

Water Extract

Acidify to pH 4.4

Protein Precipitate

Whey Solution-1

Wash twice  
with water

Acid-Precipitated Protein

Neutralize  
to pH 8.0

Phytate-Protein  
Complex

Whey Solution-2

Dialyze,  
freeze-dry

Whey Protein

FIGURE 2  
Preparation of protein fractions from dehulled defatted meal.



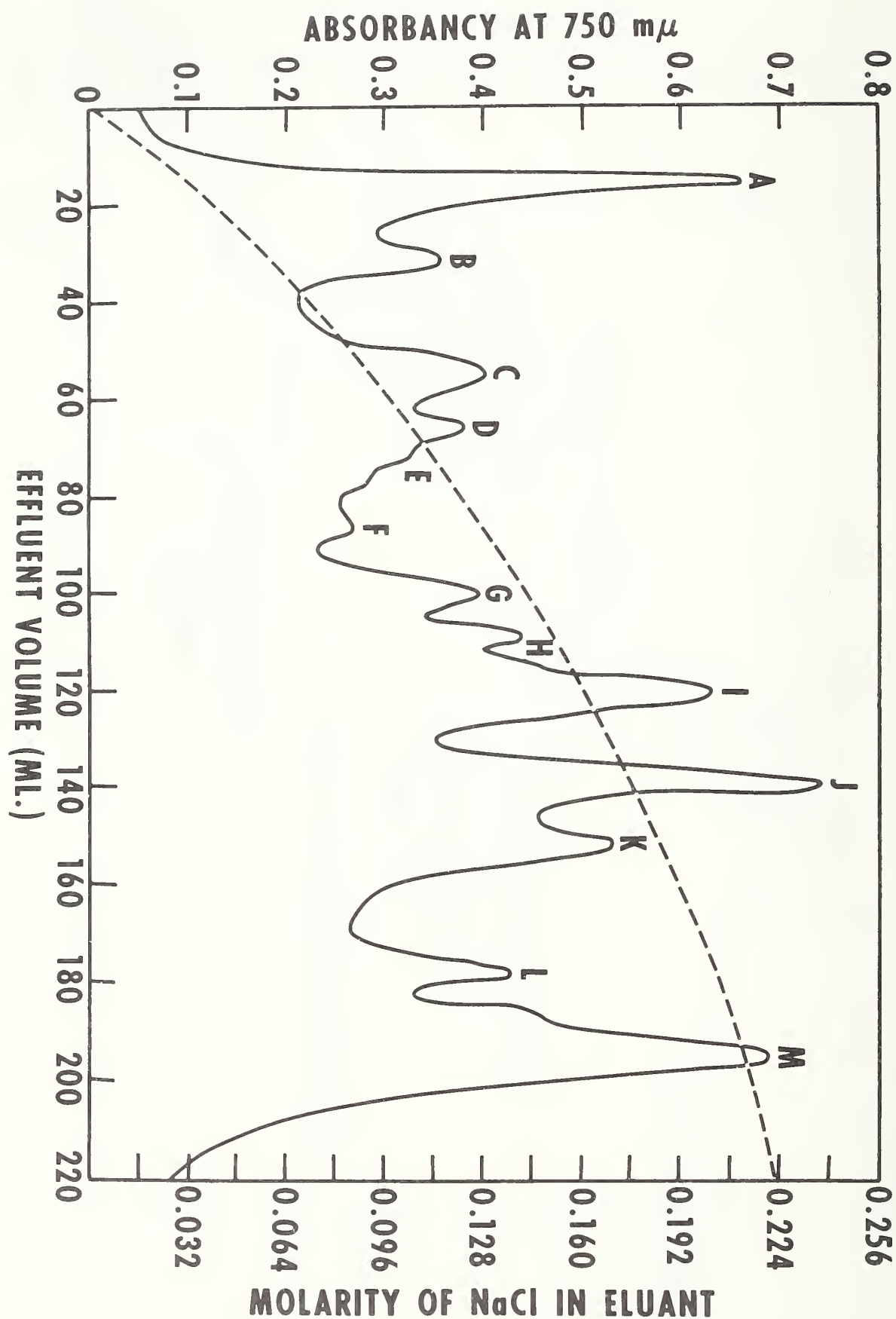


FIGURE 3  
Gradient elution of soybean whey proteins on DEAE-cellulose: effluent collected in 1 ml. fractions. Gradient limit 0 to 0.3M sodium chloride in 0.01M potassium phosphate buffer, pH 7.6. Broken line and scale at right represent sodium chloride concentrations in eluant.

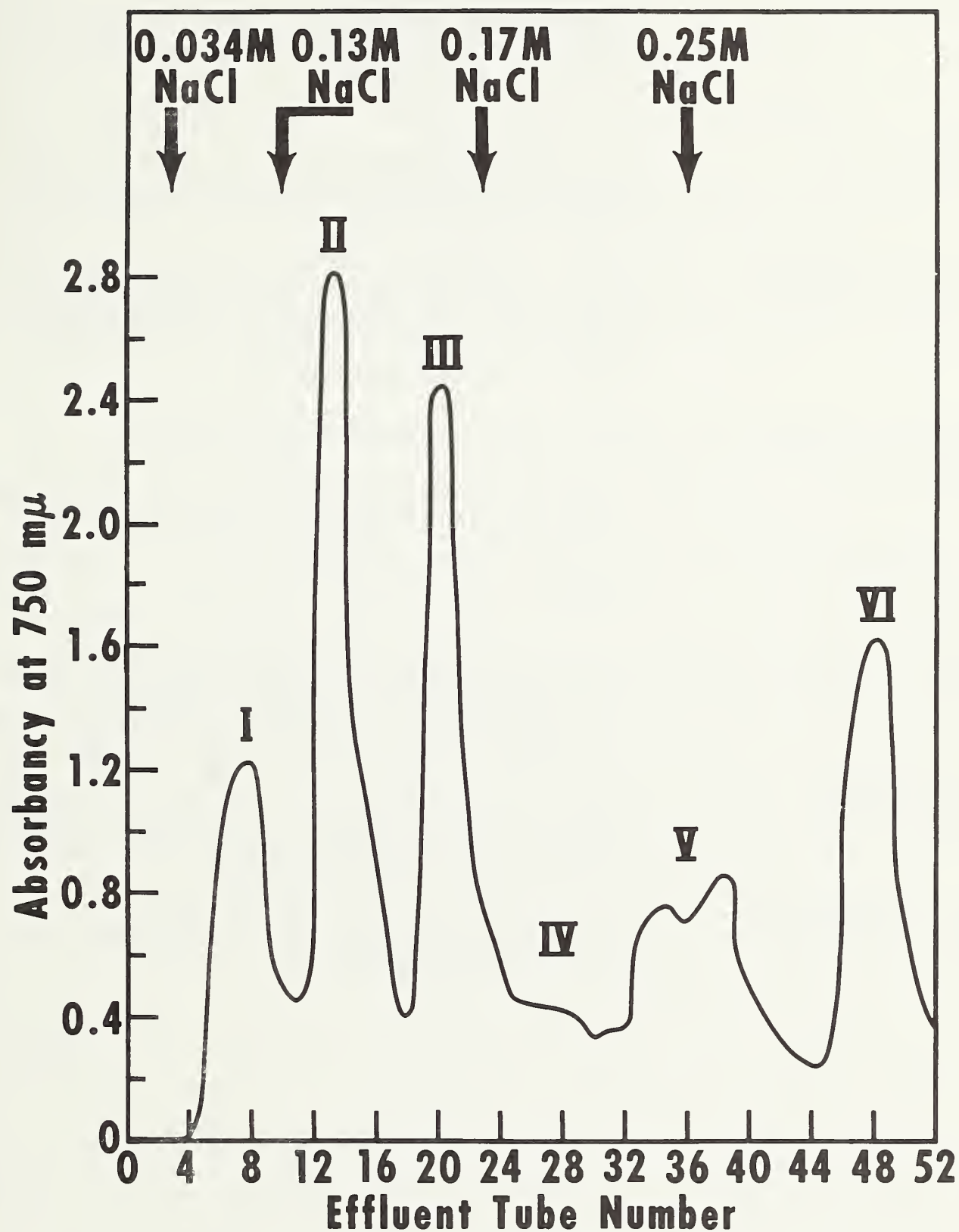


FIGURE 4  
Stepwise elution diagram of whey proteins on DEAE cellulose: effluent fraction was 10 ml.; vertical arrows indicate point of change of sodium chloride concentration in 0.01M phosphate buffer, pH 7.6.

## Discussion

- Dr. Nathan Sharon: Has a trypsin inhibitor which is also active against chymotrypsin been isolated?
- Dr. Joseph J. Rackis: We haven't tested for this activity.
- Dr. Nathan Sharon: Dr. Birk at Rehovoth has isolated an inhibitor with physical constants similar to those of fraction A<sub>1</sub>.
- Dr. Aaron M. Altschul: Could an artifact be contributed by acid treatment? This may be the reason why inhibitors made in different laboratories differ in their properties.
- Dr. Joseph J. Rackis: I think the method is reproducible.
- Dr. Nathan Sharon: I think there is a possibility of differences caused by freeze-drying, dialysis, etc.
- Dr. J. C. Perrone: Has any other substrate besides casein been used?
- Dr. Joseph J. Rackis: Yes, we are also going to set up an electro-titration using a synthetic substrate (Benzylarginylethyl ester). Calcium was used to prevent side specificities of trypsin.
- Dr. Pierre Grabar: Some substrates are attacked by all trypsins. Control of the trypsin preparations may be made by immunoelectrophoresis.
- Dr. Mark A. Stahmann: The forms of trypsin may be analogous to forms of hemoglobins.

## OILSEED ALLERGENS

Joseph R. Spies  
Allergens Laboratory  
Eastern Utilization Research and Development Division  
Washington, D. C.

### Summary

A brief description of the origin and organization of what is now the Allergens Laboratory of EURDD was given. Some basic principles of allergy and immunology, some definitions and the cutaneous and passive transfer tests used in the work were described.

The isolation and chemical and immunological properties of the principal allergen of cottonseed, CS-1A, were described. This isolation procedure was generalized by its application to other oilseeds and nuts which form a distinct clinical grouping. Almond nuts, Brazil nuts, castor beans, Barcelona and DuChilly filbert nuts, flaxseed, kapok seed and mustard seed yielded allergens chemically similar to CS-1A, but possessing their own specificities. Coconut, pecan nuts, tung nuts and English walnuts yielded no allergen by the CS-1A procedure. Peanuts, soy beans and black walnuts yielded a fraction chemically similar to CS-1A, but immunologically atypical. These allergens were classified as natural proteoses. They have the following properties: soluble in water and basic lead acetate solution, insoluble in 75% ethanol, stable to boiling water, partially dialyzable, composed of amino acids and characterized by relatively high arginine and glutamic acid contents, contain chemically combined polysaccharide, but allergenic and antigenic specificities are due to the protein components; they are immunologically distinct from other allergens and antigens in respective seeds or nuts.



The effects of the proteolytic enzymes, trypsin, chymotrypsin, pepsin and carboxypeptidase on the cottonseed allergen were discussed. Trypsin and chymotrypsin followed by trypsin destroyed allergenic activity of CS-1A. Pepsin and carboxypeptidase partially hydrolyzed CS-1A and the split products retained allergenic and antigenic activity.

Dialysis and chromatographic fractionation of CS-1A with Amberlite IRC-50 (XE-64) yielded two active fractions which were demonstrably free from each other.

A quantitative, passive transfer method using serum from a cottonseed sensitive person was devised to critically evaluate and compare these separated allergenic fractions. The method determined and compared: (1) the passive transfer inciting capacities, (2) the reagin neutralizing capacities, and (3) whether or not the specificities of two allergenic fractions were the same. The reproducibility of the method was such that twofold differences in quantity of allergen could be determined. Conclusive, confirmatory evidence was obtained with this method showing that CS-1A was a complex mixture of proteins and polysaccharidic proteins, essentially, all possessing the same allergenic specificity. It was shown by this method that  $10^{-11}$  to  $10^{-12}$  molar solutions of allergen could be detected in human test subjects.

The remarkable stability of oilseed allergens to heating in aqueous solution was discussed and the conditions of time, temperature, and pH for the inactivation of ricin and allergenic fraction CB-1A from castor beans was summarized. Both the isolated allergen and allergen in the pomace were used in this study.

The immunological and clinical response of one-hundred and sixty-one nonallergic persons to subcutaneous injection of castor bean allergen, CB-1A, was summarized.

## References

1. The Chemistry of Allergens. XI. Properties and Composition of Natural Proteoses Isolated from Oilseeds and Nuts by the CS-1A Procedure by Joseph R. Spies, E. J. Coulson, Dorris C. Chambers, Harry S. Bernton, Henry Stevens and James H. Shimp, J. Am. Chem. Soc. 73 3995 (1951).
2. The Immunochemistry of Allergens. IX. The Relationship of Carbohydrate to the Antigenic Protein from Cottonseed by E. J. Coulson, Joseph R. Spies and Henry Stevens, J. Immunol. 62 171 (1949).
3. The Chemistry of Allergens. XII. Proteolysis of the Cottonseed Allergen by Joseph R. Spies, Dorris C. Chambers, E. J. Coulson, Harry S. Bernton and Henry Stevens, J. Allergy, 24 483 (1953).
4. The Chemistry of Allergens. XIII. Ion-Exchange Fractionation of the Cottonseed Allergen and Immunological Properties of the Products by Joseph R. Spies, Dorris C. Chambers and E. J. Coulson, Arch. Biochem. and Biophysics, 84 286 (1959).
5. Quantitative Analysis of Allergens by a Passive Transfer Method as Demonstrated with Fractions of Cottonseed Allergen, CS-1A by Joseph R. Spies, Harry S. Bernton and Dorris C. Chambers, J. Allergy, 31 162 (1960).
6. Quantitative Measurement of the Migration of Intracutaneously Injected Cottonseed Allergen in Passive Transfer Studies by Joseph R. Spies, Harry S. Bernton and Dorris C. Chambers, J. Allergy, 31 175 (1960).
7. The Chemistry of Allergens. Inactivation of the Castor Bean Allergens and Ricin by Heating with Aqueous Calcium Hydroxide by Joseph R. Spies, E. J. Coulson, Harry S. Bernton, P. A. Wells, and Henry Stevens, Agric. and Food Chemistry, 10 140 (1962).
8. Response of Nonallergic Persons to Injected Castor Bean Allergen, CB-1A, by Joseph R. Spies and Harry S. Bernton, J. Allergy, 33, 73-83 (1962).

## Discussion

- Dr. J. C. Perrone: Did you use only fraction CB-1A or the whole castor bean?
- Dr. Joseph R. Spies: Both.
- Dr. J. C. Perrone: Did you use dry or wet heat?
- Dr. Joseph R. Spies: Suspensions in  $\text{Ca(OH)}_2$ . Wet heat seems to destroy activity.
- Dr. Erhard Gross: Were any of the fractions of cottonseed allergens characterized further than dialyzable and non-dialyzable?
- Dr. Joseph R. Spies: No.

PHYTOCHROME--THE CONJUGATED PROTEIN CONTROLLING  
THE PHOTORESPONSIVE GROWTH OF PLANTS

H. W. Siegelman

U. S. Department of Agriculture, Agricultural Research Service  
Crops Research Division, Plant Industry Station  
Beltsville, Maryland

Abstract

Action-spectra studies (H. A. Borthwick and S. B. Hendricks, Science 132: 1223-1228, 1960) revealed the presence of a photoreversible photoreceptor in plants, which controlled many aspects of growth and development, such as seed germination, flowering, and leaf expansion. The wavelength maxima and the photoreversibility of the photoreceptor, now called phytochrome, provided the specific properties which enabled the pigment to be detected by physical means. Phytochrome was assayed by spectrophotometric methods and isolated (W. L. Butler, et al., Proc. Nat. Acad. Sci. 45: 1703-1708, 1959). Recently, the conjugated protein has been purified by the development of large-scale protein chromatographic methods. The purification procedures consist of chromatography on diethylaminoethylcellulose, calcium phosphate, molecular sieves, and finally fractional elution of an ammonium sulphate precipitate. The photoreversible behavior of the blue purified phytochrome is clearly visible.

The nature of the chromophoric group of the protein is unknown. Limited studies suggest that the reversible absorption of the chromophore is dependent on maintaining the native structure of the protein. It is suggestive that phytochrome may be of considerable value in studies on protein structure and denaturation.



## Discussion

- Dr. Nathan Sharon: How do you account for the 20% baseline?
- Dr. Harold W. Siegelman: Some seeds do not need light at all.
- Dr. Nathan Sharon: Why wasn't lyophilization used to concentrate phytochrome?
- Dr. Harold W. Siegelman: There was a 20% loss for every freezing and thawing.
- Dr. Joe H. Cherry: Is light necessary to make phytochrome?
- Dr. Harold W. Siegelman: No. It can be synthesized in complete darkness.
- Dr. James F. Harrington: After concentration is the 1/2 life still 20 minutes?
- Dr. Harold W. Siegelman: The 1/2 life of the concentrated sample is about 10 days at 2°C. in presence of sucrose; 20 minutes at room temperature.
- Dr. Pierre Grabar: Does the pigment contain a metal?
- Dr. Harold W. Siegelman: The guess is "no" because of the extracting procedure. These proteins seem to resemble bile proteins. There are some Cu-proteins with similar spectra, but these do not show the wavelength shift.

PROTEIN BODIES, NUCLEIC ACIDS, AND ENZYMES OF PEANUT COTYLEDONS:  
CHANGES ON GERMINATION

Joe H. Cherry<sup>1/</sup>  
Horticulture Department  
Purdue University  
Lafayette, Indiana

Introduction

Seeds vary enormously in their protein content; some, such as the avocado (Persea species), contain as low as 5% protein (moisture-free basis). Others, the soybean (Glycine max) for example, contain up to 40% protein. Part of this variation is probably due to the greater quantities of biologically active proteins in some seeds as compared to others, but by far the greatest difference comes from the presence in some seeds of large quantities of so-called reserve proteins (Danielson, 1956; Steward and Thompson, 1953). These occur as globulins in some seeds, as glutelins and prolamines in others (Bondi, 1958). Thus far no enzymatic activity has been attributed to this class of proteins; their function is assumed to be primarily a source of amino acids or of nitrogen for the growing seedling.

It is not known whether all or most of these reserve proteins are located in subcellular inclusions. Some of them are regarded to be within aleurone grains; these are particularly abundant in the storage tissue of seeds which have a high protein content (Mottier, 1921). Aleurone grains are considered to be ergastic inclusions which are found in the embryo and endosperm (Esau, 1960); these have been observed by means of light microscopy. Some are composed of an amorphous protein mass enclosing crystalloids;

---

<sup>1/</sup> The research was conducted while the author was a Postdoctoral Resident Research Associate in the Seed Protein Laboratory, Southern Utilization Research and Development Division, U. S. Department of Agriculture, New Orleans 19, Louisiana.

others contain spherical bodies called globoids which may contain phytin; others are composed entirely of protein; and there are various combinations of the above-mentioned types (Guillermond, 1941). Other protein bodies (ergastic inclusions), believed to contain zein, have been detected in the maize endosperm (Duvick, 1961). As more information on the ultrastructural level is available, a different type of classification may be developed.

We have been interested in the nature and metabolism of the major seed proteins and have selected for our study the peanut (Arachis hypogaea), which contains between 20 to 25% protein in its mature cotyledons. However, as our work in the seed proteins progressed, we became interested in other cellular activities, namely nucleic acid metabolism and the glycolytic and mitochondrial enzymes. It is known that the changes in the enzymatic and mitochondrial activities during germination and development of the seedling plant do not remain constant but seem to fit a definite pattern depending on the physiological age and type of tissue. The metabolic control over this pattern of increased and decreased enzyme activities is not known. If there is a de novo synthesis of enzymes involved in the breakdown of proteins, lipids, and carbohydrates, it is reasonable that nucleic acids ultimately control their formation and possibly are involved in enzyme activation. It was of interest, therefore, to observe the cytological changes in sections of peanut cotyledons in relation to studies on the metabolism of nucleic acids and activities of various enzymes to provide additional information of the biochemical changes in the peanut cotyledon during germination.

## Materials and Methods

Virginia 56-R peanut seed<sup>2/</sup> were lightly dusted with chloranil or 2,3-dichloro-1,4-naphthoquinone and placed in vermiculite to be germinated in a dark, humid atmosphere at 30°C. Standard procedures were used for light microscopy (Sass, 1958), electron microscopy (Lund et al., 1958; Palade, 1952), protein (Lowry et al., 1951), and nucleic acid (Cherry, 1962) analysis, oxidative and phosphorylative activities of isolated mitochondria (Hanson et al., 1959), and enzyme assays (Cherry et al., 1962; Hiatt, 1961; Cooperstein and Lazarow, 1951; Kornberg and Beevers, 1957; Rao and Ramakrishnan, 1962; and Smith and Gunsulas, 1957).

## Results and Discussion

**Depletion of Storage Materials:** During germination of peanut seed and development of the seedling plant the dry weight of the cotyledon decreases 60% or about 440 mg per seed (Fig. 1). This large loss of material is attributed to the disappearance of storage reserves; one of these reserves is protein. The depletion of protein from the peanut cotyledon is shown in Fig. 1. The protein content slightly decreased the first 5 days of germination, followed by a rapid decrease in content between 6 to 9 days. By 10 days of germination about 70% of the protein was depleted with little loss thereafter. The depletion of total and cytoplasmic (debris-free) protein roughly paralleled.

**Changes in Protein Bodies:** The resting seed: The parenchyma cell of the peanut cotyledon is characterized by numerous subcellular particles which are generally spherical, ranging in size from 2-10 $\mu$  (Fig. 2a and 3).

---

<sup>2/</sup> The author is indebted to W. K. Bailey for the peanut seed used in this study.



Most of them stain from protein with Safranin O and stain heavily with osmium tetroxide. The particles isolated by Dieckert et al., <sup>3/</sup> appear by both light and electron microscopy to be similar to those shown in Fig. 2 and 3.

The isolated particles have the appearance of classical aleurone grains, inasmuch as most of them seem to contain globoids. Dieckert et al. (1962) recognized the appearance of 2 types of particles in the cotyledon on the basis of phytic acid and nitrogen contents. The aleurone grains were the classical type, and the others were simply protein bodies which showed no inclusions. It is not possible to differentiate between these 2 types merely by observations of sections of the peanut cotyledon, since some of these particles might not have been sectioned at the proper point to show globoids. For this reason in further discussion the protein particles will collectively be termed protein bodies without distinguishing between the various members.

Also visible in the electron micrographs (Fig. 3 and 5a) is a configuration that indicates a honey-comb-like structure between the bodies and connecting to the subcellular particles. This is similar to the fraction isolated by Dieckert et al. (1962) which has the appearance of a network and which has a nitrogen content of 7%.

Scattered starch grains are about the same size as the larger protein bodies ranging from 5-10 $\mu$ . These are shown in the photomicrograph (Fig. 2b) taken in polarized light. In the photomicrograph (Fig. 2a) made with ordinary light, the starch grain is seen as a dark body with a surrounding

---

<sup>3/</sup> Unpublished data from this laboratory by J. W. Dieckert, J. E. Snowden, Jr., and A. M. Altschul.

light ring. (Compare the same field made under normal [Fig. 2a] and polarized light [Fig. 2b].)

The cell wall is shown as being quite irregular with various constricted areas (Fig. 3). Apparently as a consequence of soaking the seed in cold water, the cell wall becomes separated from the cytoplasmic mass as shown by the empty space between the cell wall and the intracellular network. No holes or pits were visible in the cell wall of resting seed, but are evident at various points in walls following seed germination (see Fig. 4d and 6). The intercellular spaces are rather small in the resting seed.

Changes in Protein Bodies during Germination: Cellular and Particle Level: In the early stages of germination, the protein bodies apparently imbibe liquid, swell, and develop cavities (Fig. 4a). Many aggregate; in a cell typical of a 5-6 day seedling, one observes a smaller number of protein bodies each larger in size than those shown in the resting seed (Fig. 4b). As germination proceeds, these protein bodies become one large loose mass of small fragments, as shown in Fig. 4c, which represents cotyledonary cells typical of seedlings 7 - 8 days after the start of germination. In the later stages of germination, the cells become depleted of their storage protein, leaving small particles adjacent to the cell wall (Fig. 4d and 4e). These are small starch grains as is shown in the photograph taken in polarized light (Fig. 4f) of the same field shown in Fig. 4e. The number of large starch grains in a profile of a typical cotyledonary cell of a 14 to 16 day-old seedling is approximately the same as that of a cell of the resting cotyledon; in both instances, the average

number of large starch grains per cell profile is from 4 to 6. However, it should be noted that there is a large increase in small starch grains with germination.

Changes in the protein bodies are shown in more detail in the electron micrographs (Fig. 5). A sequence of the changes in protein bodies on germination involves swelling (Fig. 5a) and formation of cavities in the particles as shown in Fig. 5b. These continue to swell, and by extensive cavitation each protein body assumes a loose sponge-like structure as shown in Fig. 5c, which is typical of seedling 6-7 days after initiation of germination. This loose spongy structure aggregates in the center of the cell (see Fig. 4) and disintegrates into numerous fragments (Fig. 5d) from 0.5 to 2.0  $\mu$ m in length, and these continue to decrease in size as germination proceeds (10-12 days) as shown in Fig. 5c. In the last stages of germination (18-24 days) the fragments of protein bodies disappear, and only small particles are seen at the periphery (Fig. 5f).

The network seen in the cotyledon of resting seed is still visible in a section of a cotyledonary cell 2 days after planting (Fig. 5a). Thereafter, it fragments and is no longer visibly intact in the later stages of germination. The small starch grains visible in Fig. 5e and 5f are clearly seen in Fig. 5d and 5e.

The total protein content of the peanut cotyledon decreased as germination proceeded (Fig. 1); the greatest change occurred between the fourth and ninth day of germination. This corresponds to the period when the large swollen protein bodies with cavities disintegrated. After 15 days of germination only 22% of the original protein of the resting cotyledon remains.



The series of events believed to occur during germination are illustrated schematically in Fig. 6.

Tissue Level: Figure 7 shows a section of a group of cells taken 14-16 days after germination. A comparison of these with cells shown in Fig. 4 shows that within any given tissue at this age there are cells in different stages of protein degradation. Some look as though they are only in the initial stages of changes in the protein body breakdown, whereas others show evidence of being in the last stages of degradation. There seems to be a relationship between the state of the cell and its distance from the vascular bundle. Those cells which are farthest from a vascular tissue bundle show the most advanced in their changes. Cells closer to the vascular bundle are similar to the cotyledonary cells typical of seedlings at a much earlier stage of germination. It is clear, therefore, that in intermediate stages of germination there exists a heterogenous population of cells ranging in their degree of protein body degradation.

Chayen identified lysosomes in ripening strawberries and raspberries and suggested another explanation for the role of the vascular bundle in the breakdown of protein bodies. It may be that lysosomes near the vascular bundle remain intact while those farthest away break down by an anoxic effect. Thus, in the peanut cotyledon, the protein degradation may be due to lysosomal cathepsin activity. The vascular bundle would have a stabilizing effect on these organelles by providing oxygen or substrate for the maintenance of the integrity of their membrane. Hence, those nearest the vascular bundle remain intact, those farthest from the bundle might break down and release their cathepsin and other hydrolytic enzymes.<sup>4/</sup>

---

<sup>4/</sup> Personal communication by Dr. J. Chayen, Royal College of Surgeons, London.



During germination there is a large increase in the number of small starch grains per cell while the number of large ones remain about the same (Fig. 2b and 4f). This indicates an increase in starch with germination. It has been demonstrated that oilseed such as the peanut possess enzymes (Newcomb and Stumpf, 1953) for the breakdown of lipids by the  $\beta$ -oxidation pathway (Stumpf and Bradbur, 1959). Moreover, Beevers (1961) has shown that seeds undergo the glyoxylate cycle (Kornberg and Beevers, 1957) for net synthesis of carbohydrate from fatty acids. It is possible that the actual pathway in peanuts is through the intermediate formation of starch which later might be hydrolyzed to transport the carbohydrate to the growing portions of the seedlings.

In our electron micrographs (Fig. 3 and 5) there are no organelles with structural characteristics of mitochondria. Mitochondria are not observed because the tissue was stained for only 30 min; the staining time was about 1/4 that required for isolated mitochondria. Microsomes and the endoplasmic reticulum were not seen, nor were attempts made to isolate them. Nuclei can be seen in the light micrographs (Fig. 2, 4, and 6).

RNA Metabolism: Previous reports (Cherry and Hageman, 1961; Matsushita, 1958; and Ootu et al., 1953) show that as corn, rice, and bean seeds germinate there is a reduction in RNA of the storage tissue. Contrary to the findings in these seeds, Fig. 8 shows that as peanut seed germinate, the RNA content increases three-fold by 8 days. The net increase in RNA is followed by a rapid decline in content. The DNA content doubled by the tenth day, followed by a decline.

To ascertain whether a nuclease may be involved in the pattern of RNA change, the activity of RNase in the peanut cotyledon was assayed. Both the specific activity ( $\mu\text{g}$  RNA hydrolyzed/hr/mg protein) and activity per cotyledon remained constant until about 8 days of germination (Fig. 9). At this stage of germination RNase activity increased several fold, suggesting that this enzyme is involved in the in vivo degradation of RNA after 9 days of germination.

**Mitochondrial Activity:** Oxygen consumption by mitochondria from cotyledons of germinating peanuts with succinate and  $\alpha$ -ketoglutarate as substrates is shown in Fig. 10. Succinate was the most effective substrate; mitochondrial respiration on succinate sharply increased with germination reaching the peak of activity with a  $\text{QO}_2(\text{N})$  of 900 on the eighth day declining thereafter. Respiration on  $\alpha$ -ketoglutarate, the less effective substrate, increased to a  $\text{QO}_2(\text{N})$  of 300 on the tenth day followed by a decline in activity. The respiratory activity of peanut cotyledon mitochondria on these substrates closely agrees with the work of Howell (1961) on soybean mitochondria.

Previous reports (Cherry et al., 1961; Hanson et al., 1959; and Howell, 1961) have shown that the efficiency of phosphorylation by mitochondria from storage tissue decrease with germination. Table I shows that mitochondria from resting seed had a P/O ratio of zero. Mitochondria from 2-day-old cotyledons had good P/O ratios; subsequently the ration decreased with germination. By the fifteenth day the P/O ratio had decreased to zero or near zero.

**Electron Microscopy of the Mitochondrial Pellet:** The electron micrographs shown in Fig. 11 reveal that the material in the mitochondrial pellet

is heterogeneous, containing broken fragments of protein bodies and vesicular matter some of which is probably of the endoplasmic reticulum. There are few typical mitochondria in the pellet isolated from the cotyledons of resting seed (Fig. 11a). The many vesicular elements contain few inclusions, are slightly larger than normal mitochondria and appear to be joined together. The mitochondrial pellet from 2- and 5-day-old cotyledons (Fig. 11b and 11c, respectively) contain more typical mitochondria and fewer vesicular elements. A large number of typical mitochondria can be seen in the electron micrograph of the pellet from 8-day-old cotyledons (Fig. 11d). These mitochondria are about 0.6 microns in diameter and contain a dense internal structure. It is of significance that these mitochondria also have the greatest respiratory activity (Fig. 10). After 8 days of germination, the mitochondria appear to swell, show a disorganization of internal structure, and show a greater degree of mitochondrial disintegration (Fig. 11e and 11f); this increase in disorganization parallels the diminution of respiratory rate.

Enzymic Activity on Homogenates: Cytochrome oxidase and DPNH oxidase activity (units/minute) per cotyledon showed only slight changes with germination (Fig. 12a and 12b). DPNH and succinic-cytochrome reductase showed a developmental pattern of activity quite similar to that for mitochondria (Fig. 12c and 12d). In both cases the reductase activity was zero in resting seed and rapidly increased in activity with germination to its peak at 7-8 days of germination. Subsequent germination resulted in a decline in reductase activity. Resting seed possess a fairly active glucose-6-P dehydrogenase (Fig. 12e); during germination with the activity rapidly increasing to a maximum at 4-7 days followed by a sharp drop. The fact that glucose-6-P dehydrogenase is active in dry seed and reaches its maximum



level of activity earlier than the reductases and mitochondria suggests that the Warburg-Dickens pathway is operative sooner than the Embden-Meyerhof-Parnas pathway.

The specific activities (activity/mg protein) of the 5 above enzymes during germination of peanut seed show the same pattern as when the activity per cotyledon was plotted. One exception is that the oxidases appear to increase in activity with germination.

The germination of fat-containing seeds, such as those of peanuts, is accompanied by a decrease in the content of fat and an increase in carbohydrate (Murlin, 1934). Kornberg and Beevers (1957) have shown that the conversion of fat to carbohydrate is by the glyoxylate cycle. One of the key enzymes of this cycle is isocitritase; and Beevers (1961) and Marcus and Veslasco (1960) have shown that this enzyme has a developmental pattern of activity in germinating pumpkin seed similar to other enzymes reported in this paper. Therefore, it was desirable to assay the activity of isocitritase in the cotyledons of germinating peanuts. Fig. 13 shows the change in activity of this enzyme during germination. Both the activity per cotyledon and specific activity show an increase from 0-5 days followed by a decline with subsequent germination. As with glucose-6-P dehydrogenase (Fig. 12e), the resting seed contained a fairly active isocitritase and the peak activity was reached earlier than with mitochondria (Fig. 10). However, Beevers (1961) and Marcus and Veslasco (1960) have previously shown that resting pumpkin and peanut seed contain little or no isocitritase activity.

#### Summary

A study of the cytological changes of protein bodies, levels of nucleic acid and activities of several enzymes and mitochondria of the peanut cotyledon



was made during germination. The following observations were noted:

1. During the germination of peanut seed over 60% of the dry weight of the cotyledon and 70% of the protein is depleted.

2. Upon germination of peanut seed there is an ordered series of events leading to the degradation of storage protein in the cotyledonary cell. During germination the protein bodies swell and develop cavities within. Later these swollen bodies break up into many fragments which are digested and disappear. The major changes occur between 4-9 days.

3. In a given cell population there is a wide range of protein body degradation, the degree of degradation being related to the distance from the nearest vascular bundle.

4. In resting seed there is a honey-comb-like structure between and connected to the subcellular particles. After 2 days of germination this structure is no longer visibly intact.

5. RNA content of the cotyledon triples from 0-8 days of germination; subsequent germination results in a rapid loss of RNA. Concomitant with the in vivo degradation of RNA the RNase activity increases several-fold.

6. DNA content of the cotyledon doubles by the tenth day followed by a reduction in content thereafter.

7. Oxidative and phosphorylative activities of isolated mitochondria showed an increase during germination with their peak in activity occurring at about 8 days; subsequent germination resulted in a decline in activity. The P/O ratios with succinate and  $\alpha$ -ketoglutarate as substrates declined with seedling age.

8. Electron micrographs showed that the cotyledon of resting seed contain few typical mitochondria, but many vesicular membranes. During the

first 8 days of germination the mitochondria appear to increase in structure and internal organization. As the germination process proceeds the mitochondria swell and there is a large degree of disintegration.

9. DPNH cytochrome reductase, succinic cytochrome reductase, glucose-6-P dehydrogenase, and isocitritase of homogenates of cotyledonary tissue increased in activity to about the fifth to eighth day of germination followed by a rapid reduction in activity thereafter.

10. DPNH oxidase and cytochrome oxidase increased in activity with seedling age.

#### Acknowledgement

The author is indebted to Mr. Bill W. Bagley for the electron micrographs and to Dr. A. M. Altschul for advice given in the course of this study.

#### References

1. Beevers, H. 1961. Metabolic production of sucrose from fat. *Nature* 191:433-436.
2. Bondi, A. 1958. Plant proteins. P. 43-66 in: A. M. Altschul (ed.) *Processed Plant Protein Foodstuffs*. Academic Press, New York.
3. Cherry, Joe H. 1962. Nucleic acid determination in storage tissues of higher plants. *Plant Physiol.* 37:670-678.
4. Cherry, Joe H. and R. H. Hageman. 1961. Nucleotide and ribonucleic acid metabolism of corn seedlings. *Plant Physiol.* 36:163-168.
5. Cherry, Joe H., R. H. Hageman, F. I. Collins, and D. Flesher. 1961. Effects on X-irradiation of corn seed. *Plant Physiol.* 36:566-572.
6. Cherry, Joe H., R. H. Hageman, and J. B. Hanson. 1962. Effect of X-irradiation on nucleic acids in Zea mays. 11. On the level of ribonuclease activity in growing seedlings. *Radiation Research* 17: 740-751.
7. Cooperstein, S. J. and A. Lazarow. 1951. A microspectrophotometric determination of cytochrome oxidase. *J. Biol. Chem.* 189:665-670.
8. Danielson, C. E. 1956. Plant proteins. *Ann. Rev. Plant Physiol.* 7:215-236.
9. Dieckert, J. W., J. E. Snowden, Jr., A. G. Moore, D. C. Heinzelman, and A. M. Altschul. 1962. Composition of some subcellular fractions from seeds of Arachis hypogaea. *J. Food Sci.* 27:321-325.

10. Duvick, D. M. 1961. Protein granules of Maize endosperm cells. *Cereal Chem.* 38:374-385.
11. Esau, K. 1960. *Anatomy of seed plants.* Wiley, New York.
12. Guillermond, A. 1941. *The cytoplasm of the plant cell.* Chronica Botanica, Waltham, Mass.
13. Hanson, J. B., A. E. Vatter, M. E. Fisher, and R. F. Bills. 1959. The development of mitochondria in the scutellum of germinating corn. *Agron. J.* 51:295-301.
14. Hiatt, A. J. 1961. Preparation and some properties of soluble succinic dehydrogenase from higher plants. *Plant Physiol.* 36:552-557.
15. Howell, R. W. 1961. Changes in metabolic characteristics of mitochondria from soybean cotyledons during germination. *Physiol. Plantarum.* 14:89-97.
16. Kornberg, H. L., and H. Beevers. 1957. The glyoxylate cycle as a stage in the conversion of fat to carbohydrate in castor beans. *Biochem. Biophys. Acta* 26:531-537.
17. Lowry, O. H., N. J. Rosebough, A. L. Fair, and R. J. Randall. 1951. Protein measurement with the Folin-phenol reagent. *J. Biol. Chem.* 193:265-275.
18. Lund, H. A., A. E. Vatter, and J. B. Hanson. 1958. Biochemical and cytological changes accompanying growth and differentiation in the roots of Zea mays. *J. Biophys. Biochem. Cytol.* 4:87-98.
19. Matsushita, S. 1958. Studies on the nucleic acids in plants. III. Changes of the nucleic acid contents during the germination stage of the rice plant. *Memoirs of the Research Institute for Food Sci., Kyoto University* 14:30-32.
20. Marcus, A. and J. Veslasco. 1960. Enzymes of the glyoxylate cycle in germinating peanuts and castor beans. *J. Biol. Chem.* 235:563-567.
21. Mottier, D. M. 1921. On certain plastids, with special reference to the protein bodies of Zea, Ricinus, and Conopholis. *Ann Botany (London)* 35:349-369.
22. Murlin, J. R. 1934. The conversion of fat to carbohydrate in the germinating castor bean. I. The respiratory metabolism. *J. Gen. Physiol.* 17:283-302.
23. Newcomb, E. H., and P. K. Stumpf. 1953. Fat metabolism in higher plants. I. Biogenesis of higher fatty acids by slices of peanut cotyledons in vitro. *J. Biol. Chem.* 200:233-239.
24. Oota, Y., R. Fugii, and S. Osawa. 1953. Changes in chemical constituents during the germination of a bean, Vigna sesquipedalis. *J. Biochem. (Tokyo)* 40:649-661.
25. Palade, G. E. 1952. A study of fixation for electron microscopy. *J. Exp. Med.* 95:285-298.
26. Ragland, T. E., and D. P. Hackett. 1961. The intracellular localization of some oxidative activities in etiolated pea stems. *Biochem. Biophys. Acta* 54:577-580.
27. Rao, N. A. N., and T. Ramakrishnan. 1962. The interaction of glyoxylate with cysteine and its application to the assay of isocitri-tase and of transaminases involving glyoxylate. *Biochem. Biophys. Acta* 58:262-265.

28. Sass, J. E. 1958. Botanical microtechnique, 3rd. ed., Iowa State College Press, Ames, Iowa.
29. Smith, R. A., and I. C. Gunsalus. 1957. Isocitritase: Enzyme properties and reaction equilibrium. J. Biol. Chem. 299:305-319.
30. Steward, F. C., and J. F. Thompson. 1953. Proteins and protein metabolism in plants. P. 513-394 in: H. Neurath and K. Bailey (eds.), The Proteins, Vol. II. Academic Press, New York.
31. Stumpf, P. K., and C. Bradbur. 1959. Fat metabolism in higher plants. Ann. Rev. Plant Physiol. 10:197-222.



Table I

Phosphorylation (P/O ratios)\* by mitochondria  
from cotyledons of germinating peanuts

Age	Substrate**	
	Succinate	$\alpha$ -ketoglutarate
0	0	0
2	0.53	1.85
5	0.49	1.57
8	0.43	1.13
15	0.08	0

\* P/O = moles of inorganic phosphorous esterified per  
atom of oxygen consumed.

\*\* Twenty  $\mu$  moles succinate plus 10  $\mu$  moles pyruvate  
and 40  $\mu$  moles  $\alpha$ -ketoglutarate were used as substrate.

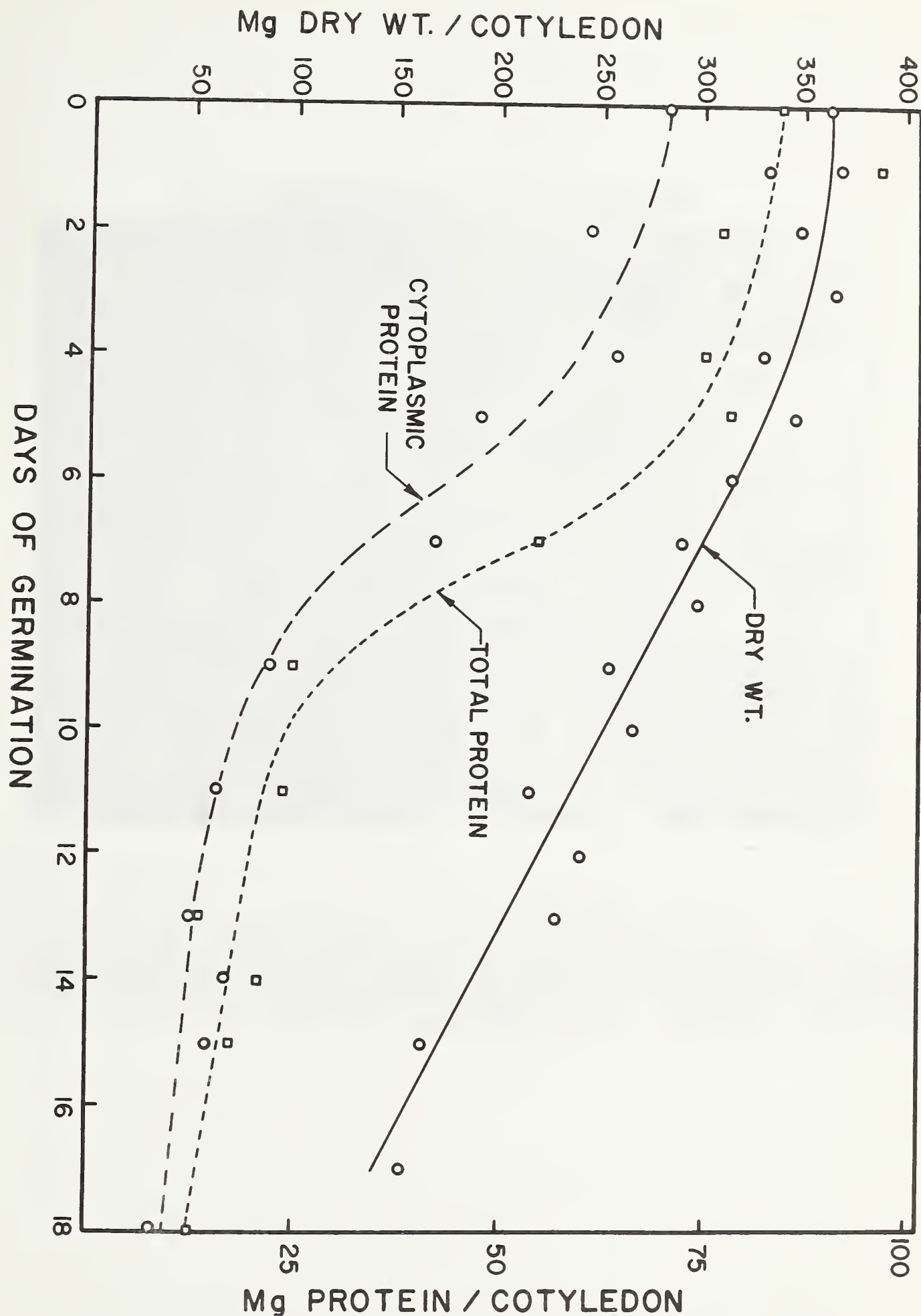


Fig. 1

Depletion of storage protein and dry weight in the peanut cotyledon during germination. Total and cytoplasmic protein was obtained by analysis on the total homogenate and the debris-free (nuclei-free) homogenate, respectively.

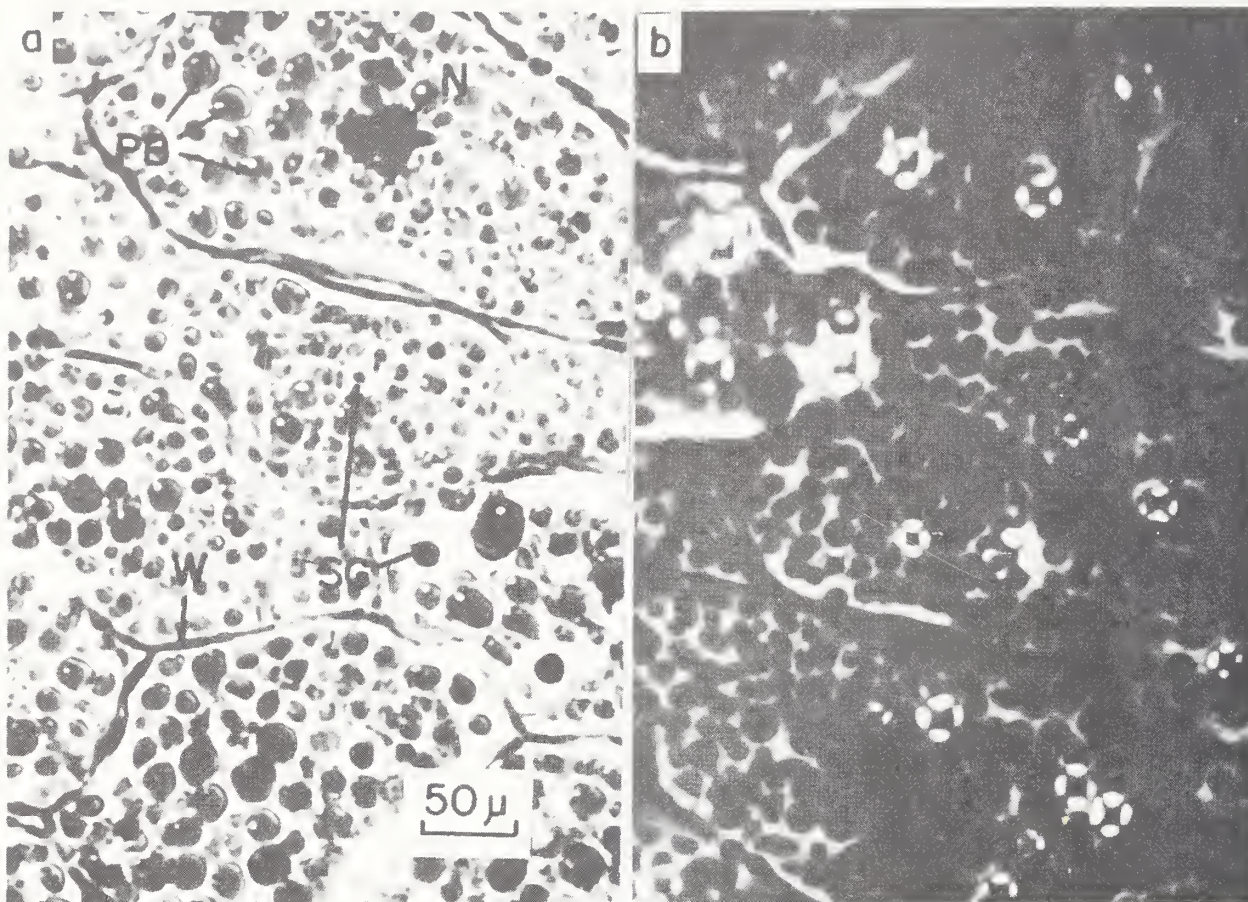


Fig. 2

Photomicrographs of cotyledonary cells of resting seeds. Many protein bodies (PB) fill these large cells shown in 2a; the thick cell walls (W) and a nucleus (N) are shown. Starch grains (SG) are shown in 2b which is of the same field as 2a taken in polarized light. X 500.



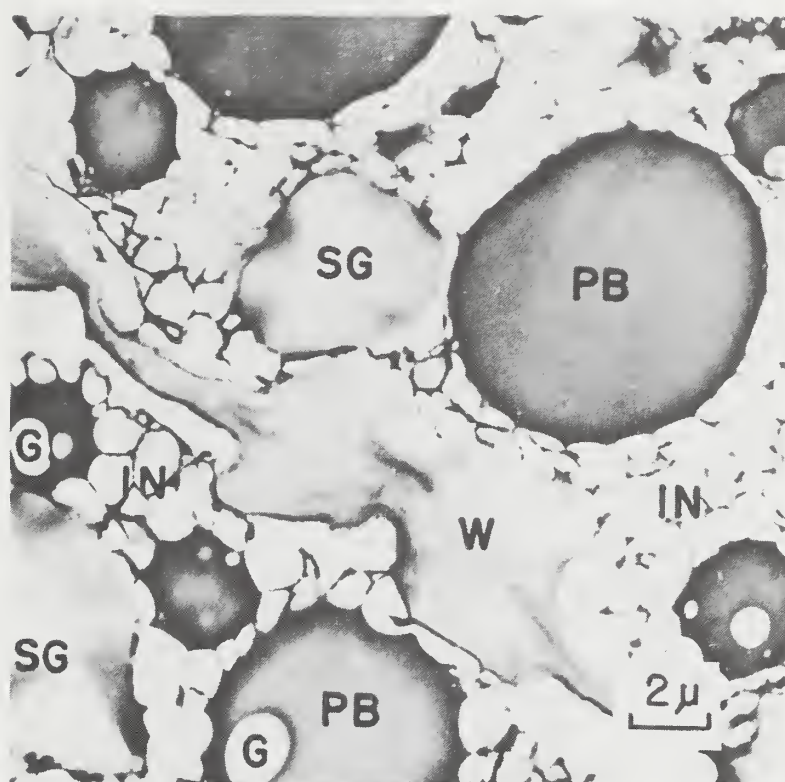


Fig. 3

A typical low-power electron micrograph of a portion of a cotyledonary cell of resting peanut seeds. The protein bodies (PB) appear spherical and very dense. Small protein bodies have inclusions which are probably globoids (G). Starch grains (SG) and the cell (W) are present. An intracellular network (IN) which appears to be three-dimensional is shown between and connecting the various subcellular particles. X 4,500.



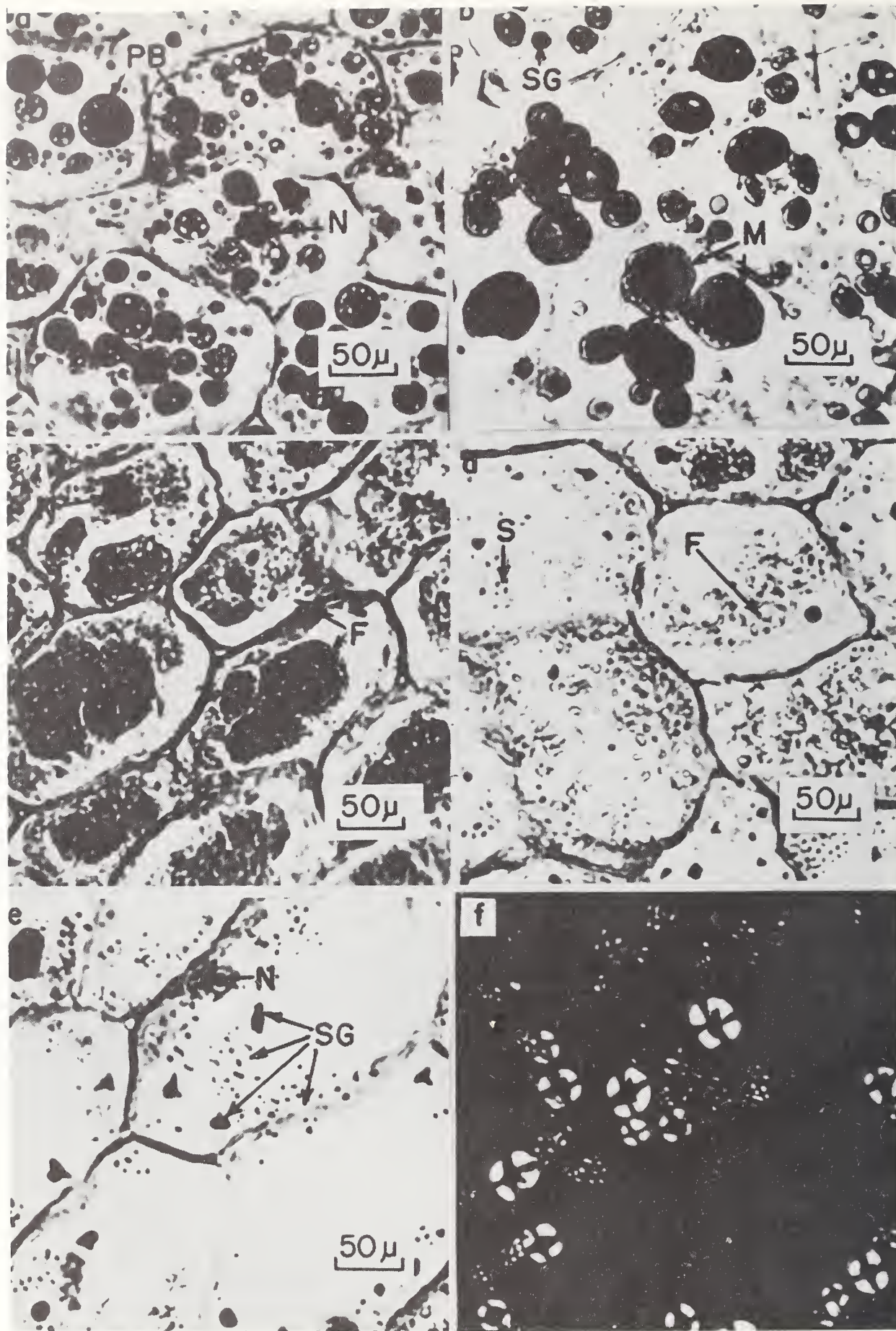


Fig. 4

Changes in cotyledonary cells during the germination of peanut seed as revealed by optical microscopy. The light micrographs show typical cells of peanut cotyledons after the following days of germination: 2-4 days, 4a; 5-6 days, 4b; 7-8 days, 4c; 10-12 days, 4d; and 14-16 days, 4e. The protein bodies (PB) appear to aggregate and to swell during germination. The large loose mass (M) of protein then breaks into small fragments (F). Cells typical of the later stages of germination (4e) appear to contain little protein, but there are many small starch grains (SG) as revealed by polarized light (4f). Several nuclei (N) are shown. X 500.



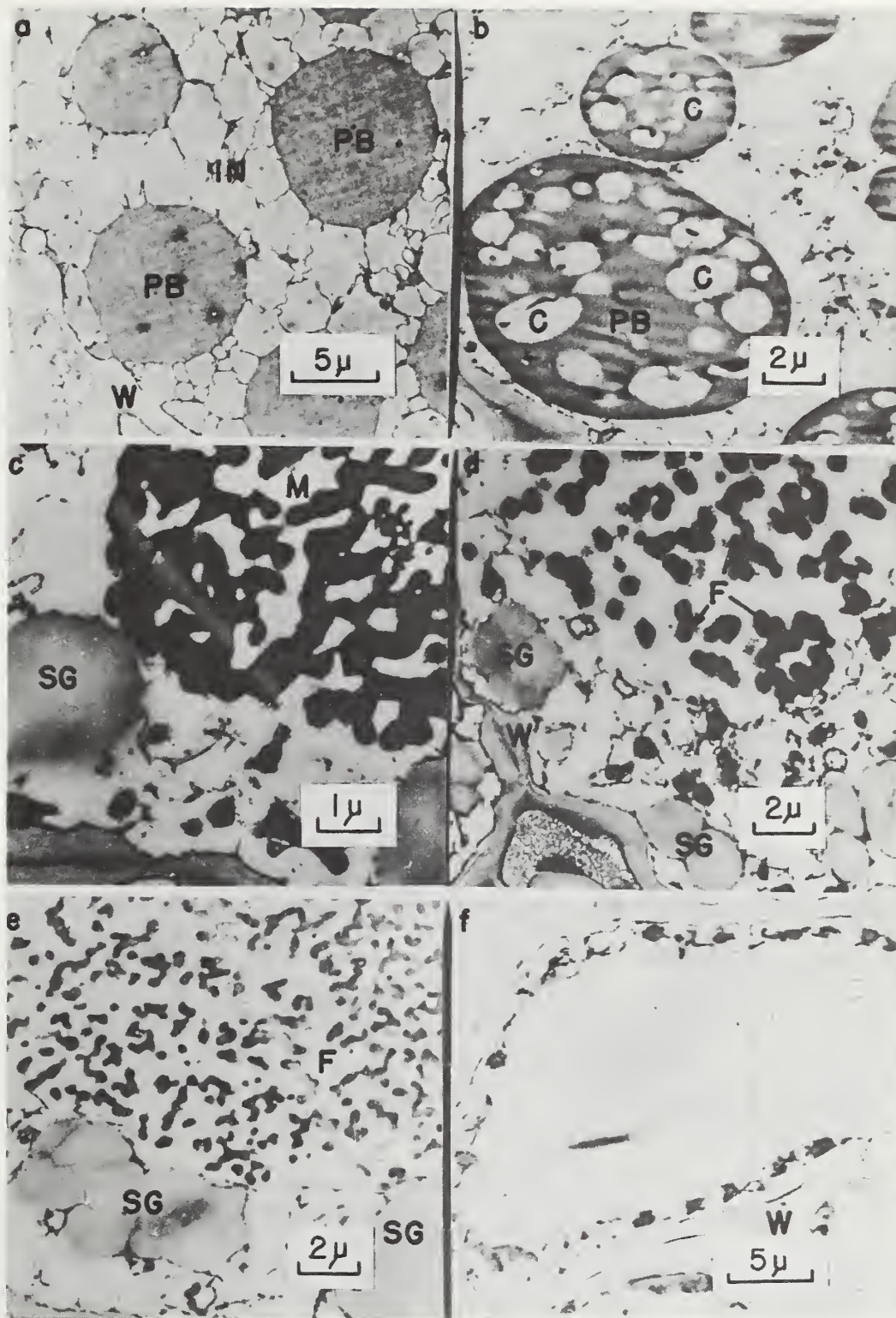


Fig. 5

Changes in protein bodies at various stages of germination as revealed by electron microscopy. The days of germination and magnification for each stage are as follows: 5a, 2 days X 2,700; 5b, 4-5 days X 4,500; 5c, 6-7 days X 10,000; 5d, 8-9 days X 6,400; 5e, 10-12 days X 6,400; and 5f, 18-24 days X 2,700. Slightly swelled but dense protein bodies (PB) which are typical of two days of germination (5a) develop cavities (C) and assume a loose sponge-like mass (M) during germination. This mass of protein subsequently disintegrates into numerous fragments (F) and disappears. The intracellular network (IN) is shown in 5a. Several starch grains (SG) and proteins of the cell walls (W) are present. The tissue shown in 5a was stained with  $\text{OsO}_4$ , poststained with  $\text{KMnO}_4$ , and embedded in Epon-Araldite; all other tissue was fixed in formalin-acetic acid-ethanol, stained in  $\text{OsO}_4$ , and embedded in methacrylate.



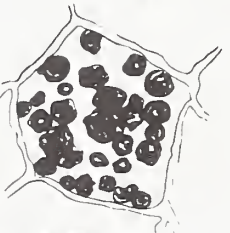

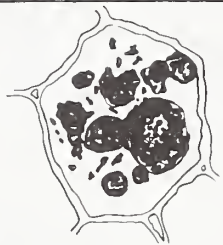

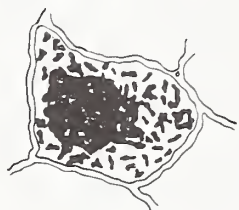



STAGE OF DEGRADATION (DAYS OF GERMINATION)	SERIES OF EVENTS		RELATIVE SIZE ( $\mu$ ) PROTEIN BODY OR FRAGMENT
	CELLULAR LEVEL	PROTEIN BODY OR FRAGMENT	
1 (0 DAY)			2-8
2 (4-5 DAY)			4-15
3 (6-7 DAY)			6-18
4 (8-9 DAY)			0.5-2.0
5 (10-12 DAY)			0.2-1.0

Fig. 6

Scheme of protein body degradation in cotyledonary cells of peanuts during germination. Five typical stages of change in the protein bodies are represented, both on the cellular and protein body or fragment levels. The relative size of the protein bodies or fragments at the various stages of degradation is given in the right-hand column.



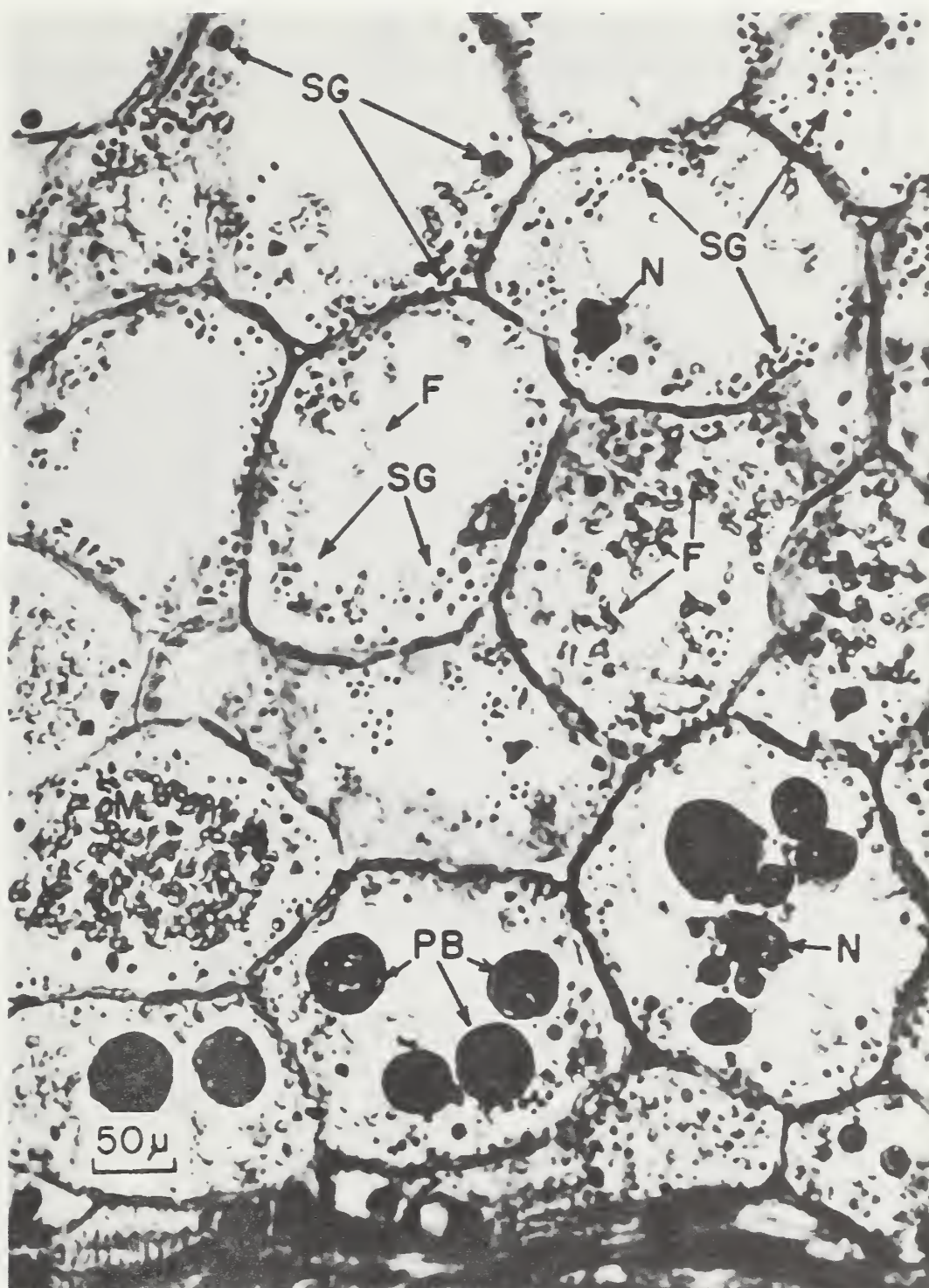


Fig. 7

The relation of cellular protein body degradation to the nearest vascular bundle (VB) is shown in a typical photomicrograph of a group of cells from a cotyledon 10-12 days after planting. The cells adjacent to the vascular bundle (bottom of photograph) have protein bodies (PB) which appear as though they are in the initial stages of breakdown. One cell which is only removed from the vascular bundle by a single cell is typical of an intermediate stage with a sponge-like mass. Other cells show small fragments (F) while others further away appear empty of protein. Empty cells shown at the top of the figure have many small starch grains (SG). A few nuclei (N) are shown. X 500.



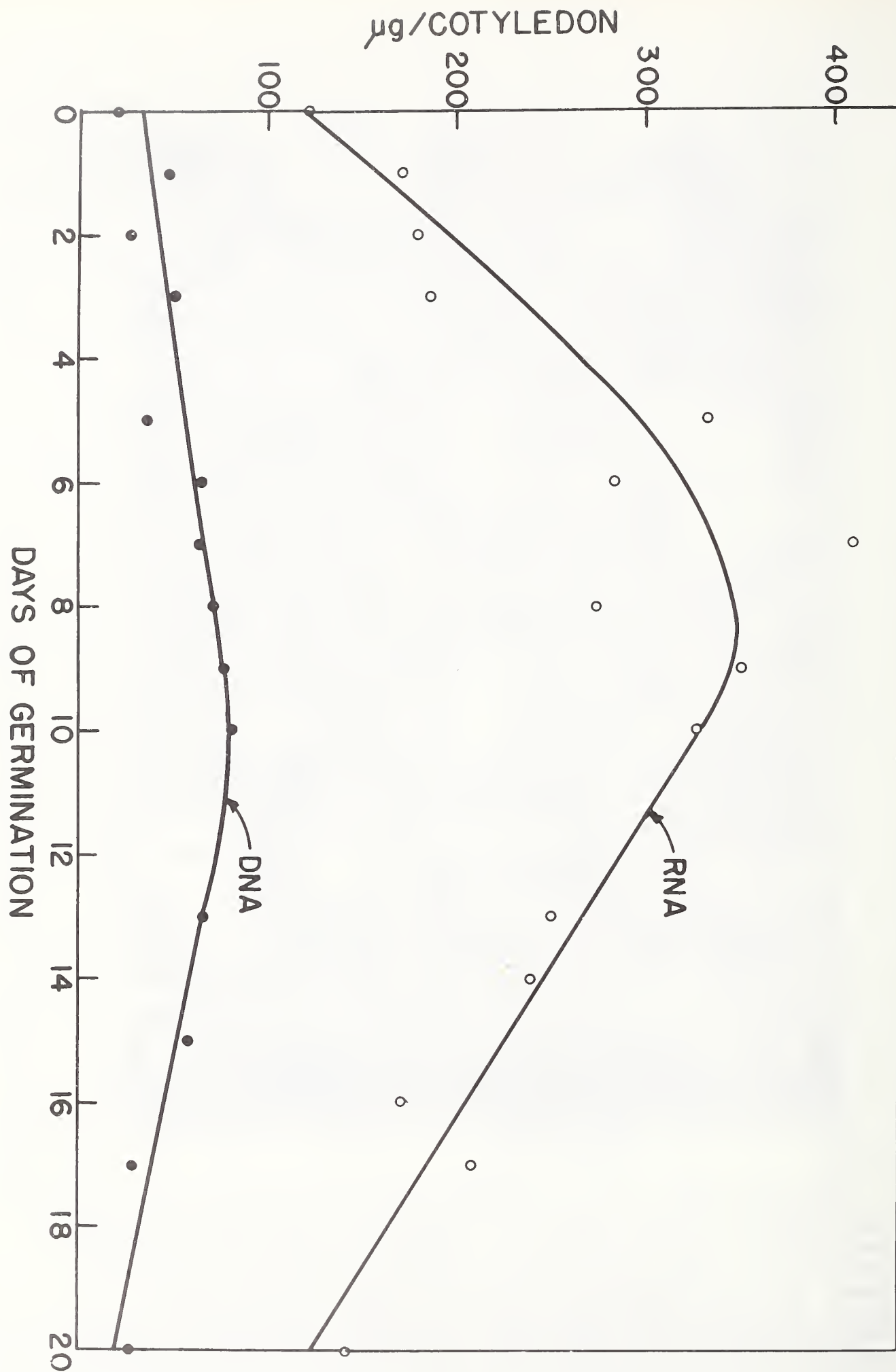


Fig. 8

Changes in nucleic acid (RNA and DNA) content in the peanut cotyledon during germination.

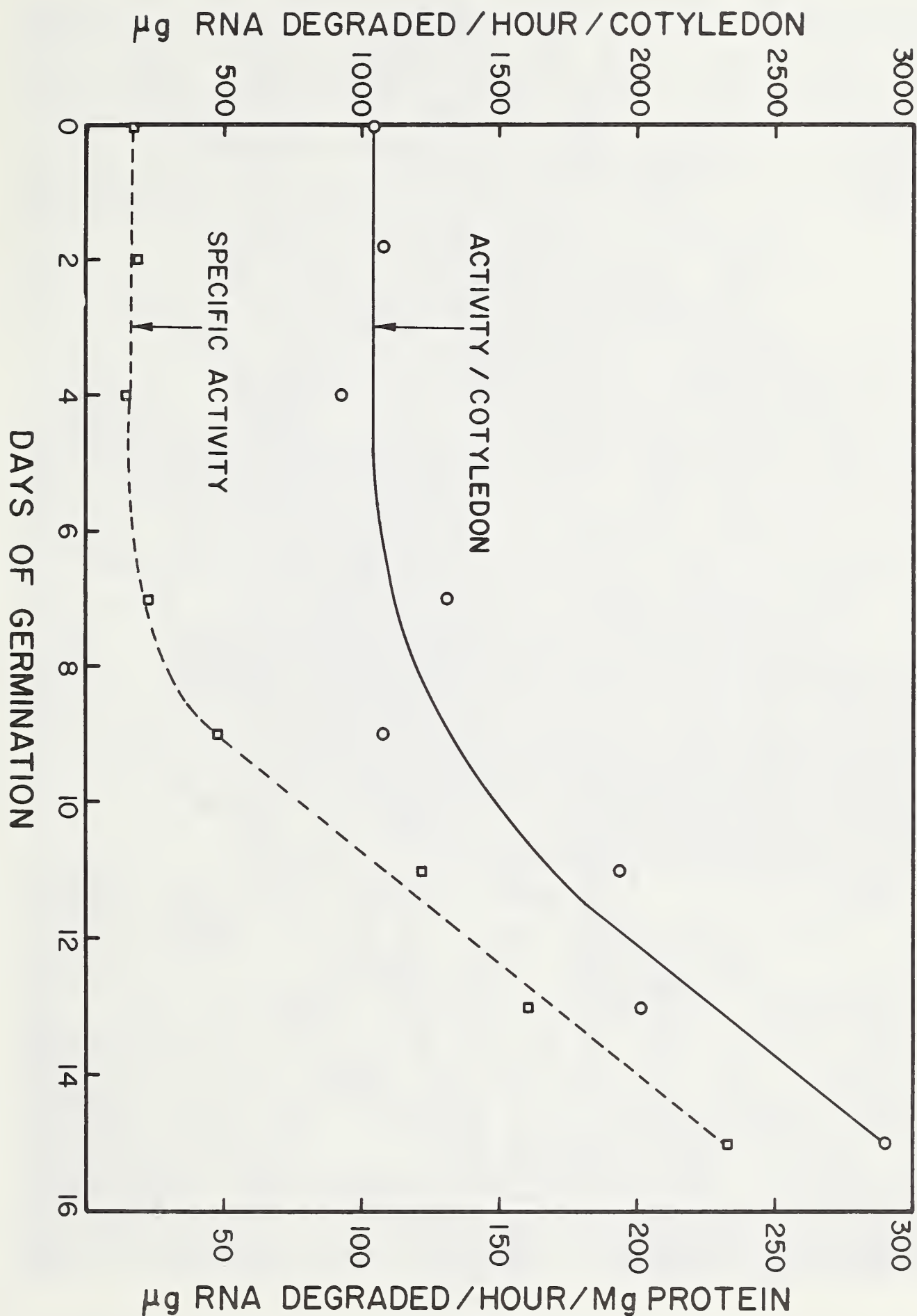


Fig. 9

Changes in RNase activity in cotyledons of germinating peanuts. Activity ( $\mu\text{g}$  RNA hydrolyzed/hr) per cotyledon and specific activity ( $\mu\text{g}$  RNA hydrolyzed/hr/mg protein) are plotted.

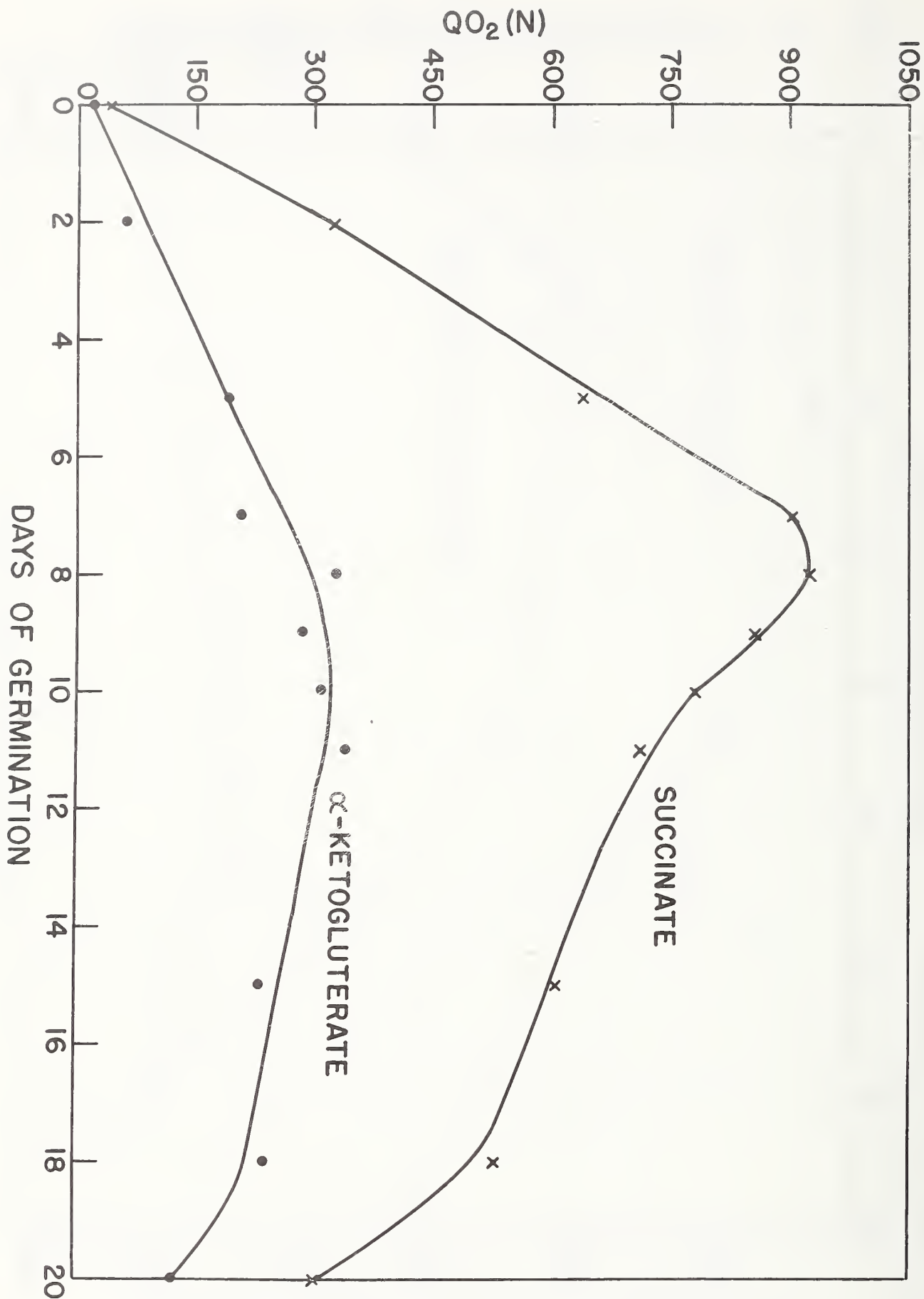


Fig. 10

Oxygen consumption by mitochondria from cotyledons of germinating peanuts with the substrates indicated. Ten  $\mu$  moles pyruvate was added to the vessels containing succinate to "spark" succinate oxidation.  $QO_2(N)$  is the  $\mu$  liters of  $O_2$  consumed per hour per mg mitochondrial  $N_2$ .



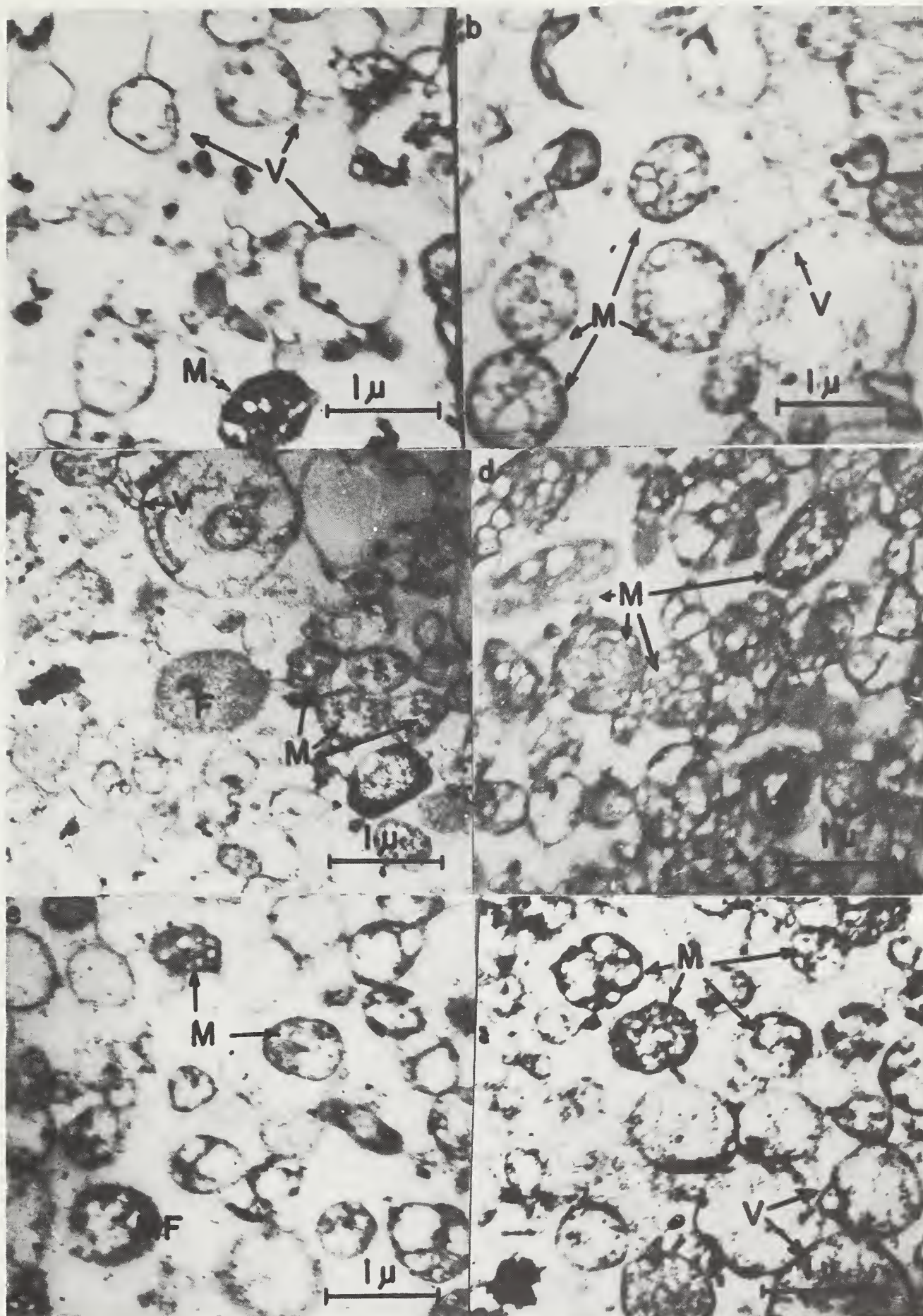


FIG. 11

Electron micrographs of once-washed mitochondria isolated from peanut cotyledons. Micrographs of the mitochondrial pellet from cotyledons after 0, 2, 5, 8, 12, and 16 days of germination are shown in a, b, c, d, e, and f, respectively. The magnification in all micrographs is 18,000 X. M=mitochondria, V=vesicular elements, F=fragment of protein body, MF=mitochondrial fragment.



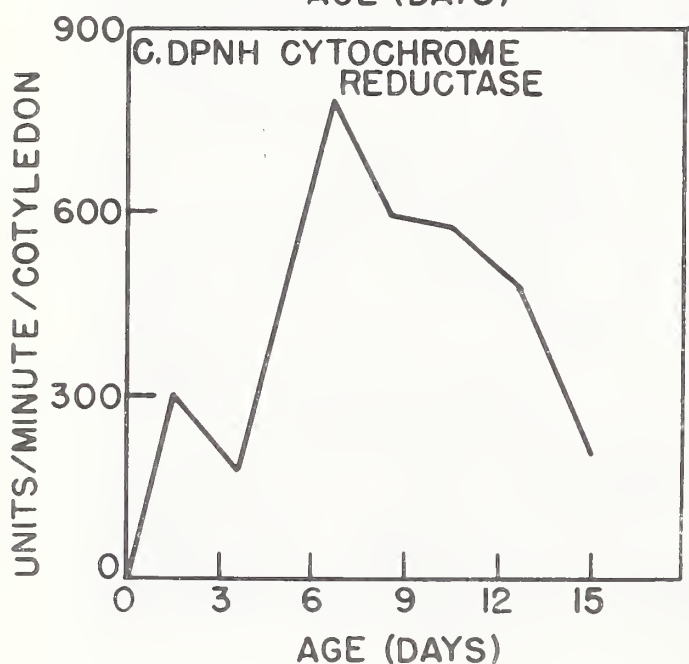
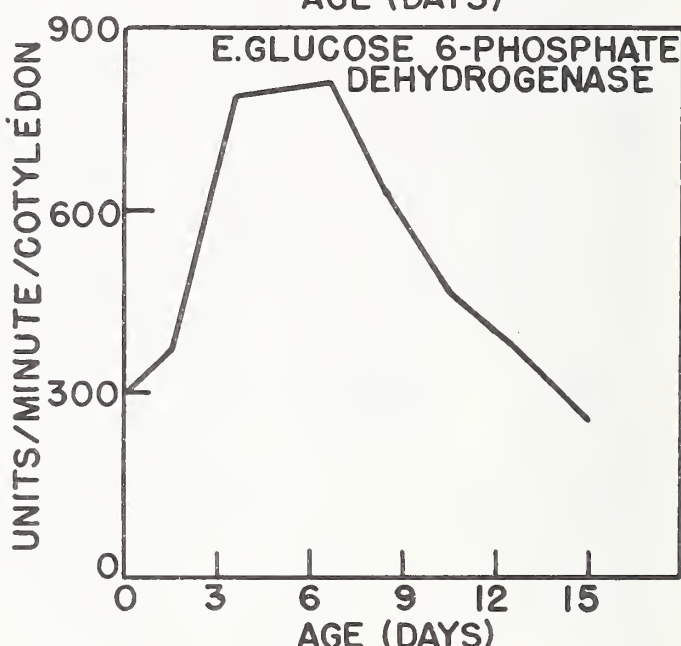
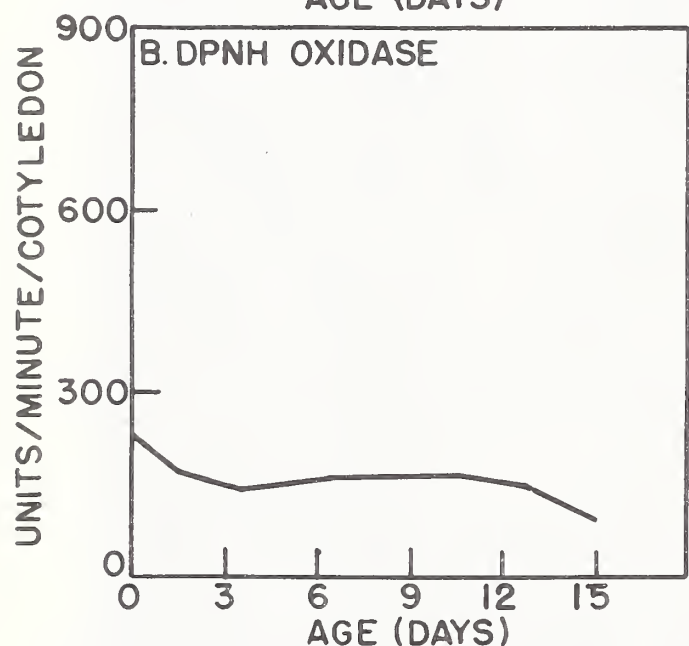
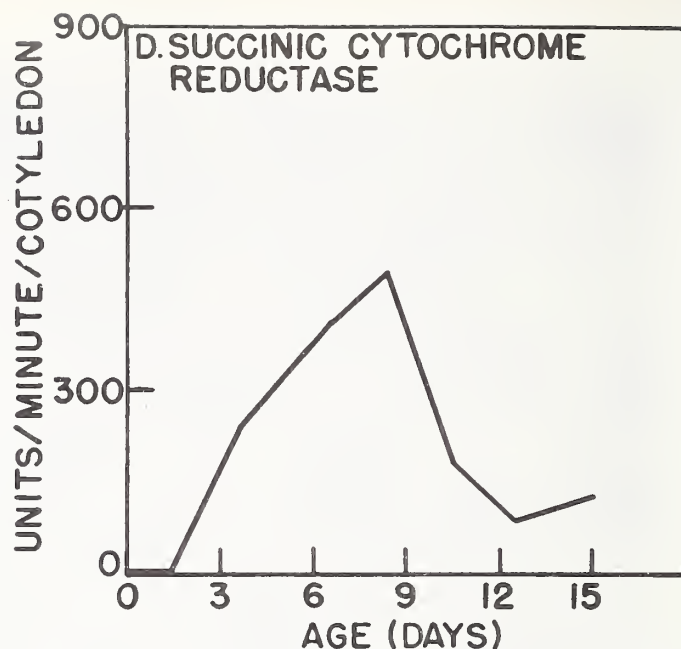
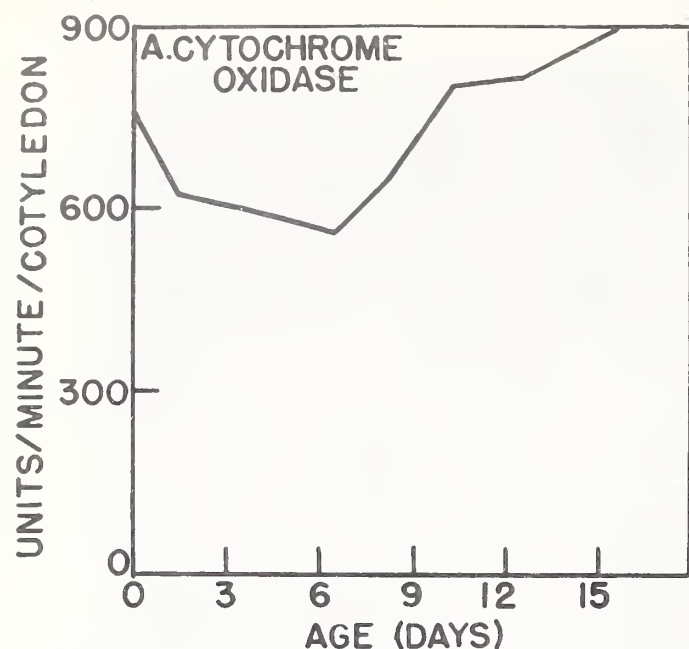


Fig. 12

Changes in activities per cotyledon of various enzymes of germinating peanuts. Homogenates were assayed for the enzymes indicated and a unit of activity expressed as a change in optical density of 0.01.

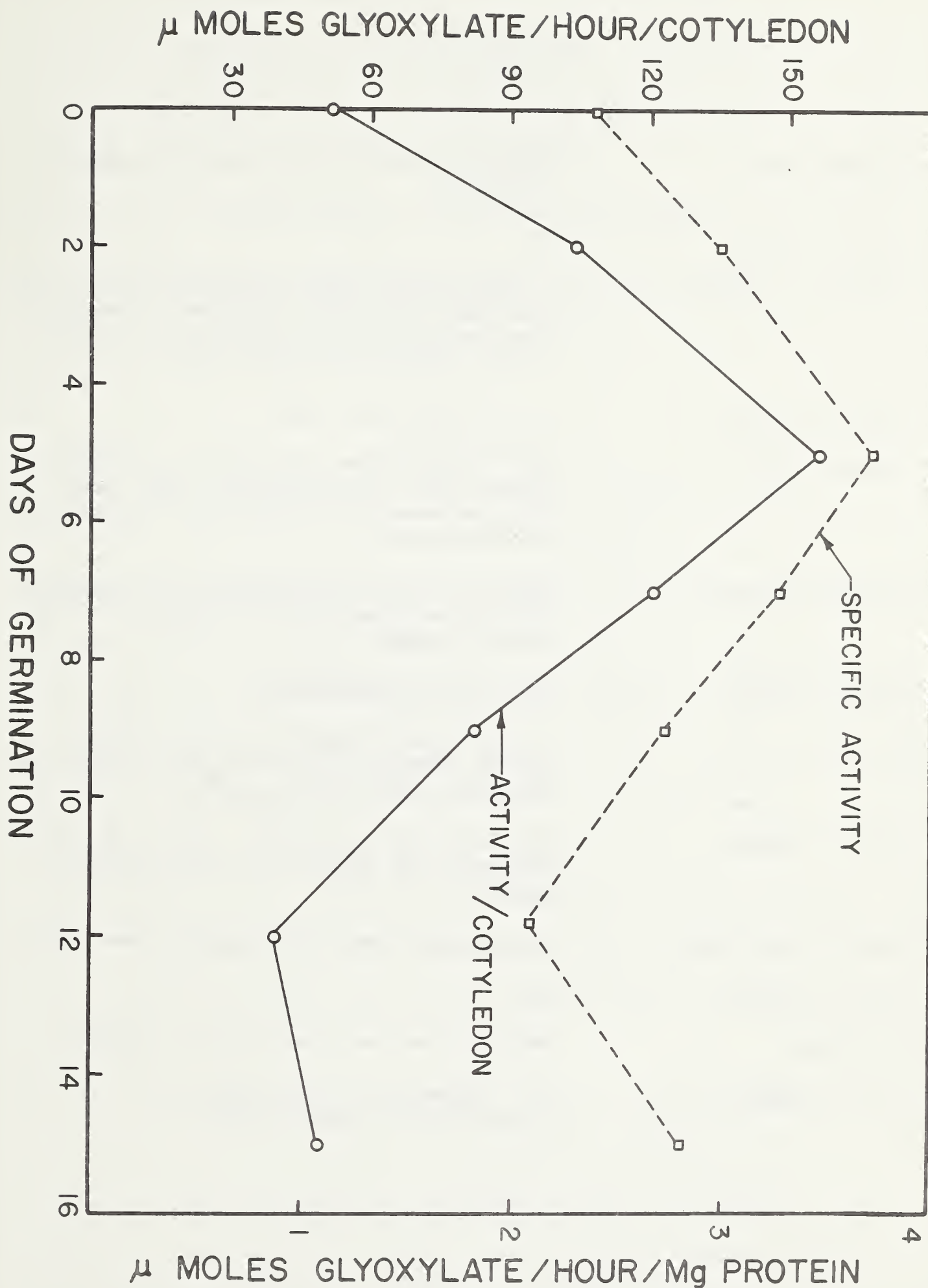


Fig. 13

Changes in isocitritase activity from cotyledons of germinating peanuts. Activity per cotyledon and specific activity are plotted.

## Discussion

- Dr. Te May Ching: Have you seen any mitochondria in peanut cotyledons?
- Dr. Joe H. Cherry: No.
- Dr. Robert W. Howell: I assume these seeds were germinated in the dark under standard conditions. How would these results compare with results under normal germinating conditions?
- Dr. Joe H. Cherry: I would guess the same.
- Dr. Abraham Marcus: There is an enzyme development at higher temperatures not obtained at lower ones. The ones seen developing at all temperatures are functional.
- Dr. Irvin E. Liener: Have you found any increases in proteolytic activity accompanying the breakdown of the protein bodies?
- Dr. Joe H. Cherry: This was not determined.
- \_\_\_\_\_:
- Would the mitochondria be in a shrunken state? Where do they come from? Where is the DNA? Where is the phytin?
- Dr. Joe H. Cherry: The DNA is in the nucleus, and according to Dieckert, the phytin is in the aleurone grains.
- Dr. Lawrence Y. Yatsu: Did you ever see chloroplasts, grana, etc.?
- Dr. Joe H. Cherry: No.
- Dr. A. Raspet: Have you ever seen any mitotic figures?
- Dr. Joe H. Cherry: No. There is no cell division in the cotyledons from etiolated plants.

Enzyme Formation in the Cotyledons and  
Endosperm of Germinating Seeds (Including  
an Examination of the Sub-cellular Structure  
of Pea Cotyledons)<sup>1</sup>

J. E. Varner and George Schidlovsky

RIAS, 7212 Bellona Ave., Baltimore, Md.

Introduction

The readily observable metabolic decline of the cotyledons and endosperm of certain germinating seeds is preceded and accompanied by the formation of enzymes which participate in bringing about this decline. Our research on these cells is proceeding on the hypothesis that even in these senescent cells metabolism is subject to close regulation and that their senescence is in fact simply the final phase of their differentiation. In this view the problem of the timing of the formation of these enzymes and of their control is no different in principle from the problem of timing and control in any phase of differentiation.

As a part of our approach to this problem we are delineating the structural and functional changes which occur in the cotyledons of germinating peas. As might have been expected from earlier reports that cotyledon cells are capable of oxidative phosphorylation (1), incorporation

---

<sup>1</sup>This work was supported in part by grant No. A-5708 from the National Institutes of Health.



of labeled amino acids into protein (1), and de novo synthesis of certain enzymes (1,2), the first examination of pea cotyledon cells by electron microscopy showed that the cells contain all of the structures normal to plant cells - a nucleus, mitochondria, endoplasmic reticulum, golgi bodies, chloroplasts and starch grains (3). In addition, a major fraction of the cell volume is occupied by roughly spherical bodies about two microns in diameter with no visible internal structure. These structures are easily isolated as a pellet following centrifugation of a pea cotyledon homogenate and contain most - perhaps all - of the reserve globulins of the seed (3). These protein bodies appear during the maturation of the seed and disappear gradually during normal germination. The presence of similar protein bodies in peanut cotyledons has already been reported (4,5,6).

The question of de novo synthesis vs. activation of pre-existing proteins as an explanation for the observed increases in enzymatic activities has been approached by the use of DEAE columns to separate the proteins of cotyledon extracts following incubation of the excised cotyledons in  $^{14}\text{C}$ -labeled amino acids. There are at least five separate peaks labeled. One of these is associated with a pyrophosphatase which increases greatly during germination. Another is associated with a  $\text{Mg}^{++}$ -requiring phosphatase which also

increases several fold during germination. Preliminary experiments indicated that  $\alpha$ -amylase of peas increases by de novo synthesis and that this synthesis is triggered off by gibberellic acid (7). For an intensive study of the problem of gibberellic acid control of  $\alpha$ -amylase formation, we have turned to barley endosperm because much of the physiology of this relationship has already been worked out (8,9,10).

Experiments to date indicate that this gibberellic acid triggered increase in  $\alpha$ -amylase is due to de novo synthesis which occurs in the aleurone cells.

#### Material and Methods

General. In all studies with peas the same variety (*Pisum sativum*. var. Early Alaska) was used. For the study of the development of the protein bodies during maturation of the pea seeds, the peas were grown in soil in a growth chamber having a light intensity of about 6,000 foot-candles with a day length of 14 hours and day and night temperatures of 23 and 17 C respectively. For the study of the changes in the protein bodies during germination, the peas were germinated in soil in the growth chamber under the same conditions as above. For isolation of protein bodies, for enzymic assay and for the labeling experiments, the peas were soaked in 1% sodium hypochlorite for 30 minutes, rinsed in sterile distilled water and germinated in moist sand in sterile Petri dishes. For chemical and enzymatic

analysis, the excised cotyledons were ground with sand in 10 mls. of 0.10 M Tris (pH 7.1) with a mortar and pestle at 0-5 C. The homogenate was centrifuged at 1500 x g for 10 minutes. The light green precipitate overlying the firm starch pellet was removed with a spatula. This constitutes the pellet fraction of Fig. 4.

The supernatant fraction was further centrifuged at 144,000 x g for 60 minutes. The 144,000 x g supernatant fraction was dialyzed against 0.01 M Tris (pH 7.8). A separate pellet fraction prepared as above was extracted at room temperature for 4 hours with 0.2 M NaCl and 0.05 M Tris (pH 7.8), centrifuged at 1500 x g. The 1500 x g supernatant fraction was dialyzed against 0.01 M Tris (pH 7.8) to the point of incipient precipitation, then added to a DEAE column. The whole extract was prepared by grinding pea cotyledons in a mortar and pestle with 0.2 M NaCl, 0.05 M Tris (pH 7.8) and continuing the extraction for 4 hours. This extract was then dialyzed in preparation for separation on a DEAE column. The globulin fraction was prepared from the whole extract according to Danielsson (11). Pyrophosphatase activity was determined by incubating a 1.0 ml. aliquot of each fraction for 60 minutes at 30 C with 300  $\mu$ moles acetate-Tris buffer (pH 5.5) and 20  $\mu$ moles of pyrophosphate in a final volume of 4.0 ml. Adenosine triphosphatase activity was determined at pH 7.1 in the

presence of  $Mg^{++}$  ions (2).

For the experiments with barley endosperm, hull-less barley (*Hordeum Vulgare*, Var. Himalaya) seeds were cut in half (at right angles to the major axis) and the embryo half discarded. The endosperm half was sterilized by soaking in 1:5 Chlorox for 10-20 minutes, rinsed in sterile water and transferred to moist, sterile sand in covered Petri dishes. After three or four days at 20-25 C, the half-seeds were transferred to 25 ml. Erlenmeyer flasks (10-20 half-seeds per flask) containing 2.0 ml. of 0.001 M acetate (pH 4.8) only or buffer plus  $10^{-6}$  M gibberellic acid (Merck, mixed practical) and incubated at 25 C overnight with rapid shaking to provide aeration. The  $\alpha$ -amylase activity was followed by the method of Shuster and Gifford (12). The 2.0 ml. solution surrounding the half-seeds plus a 3.0 ml. rinse was assayed separately from the distilled water (5.0 ml.) extract of the half-seeds (ground with sand in a mortar). A volume of 0.02-0.10 ml. incubated with 1.0 ml. of  $H_2O$  + 1.0 ml. of the starch solution for 5 minutes at 20 C is sufficient to give an easily measurable change in the starch-iodine color. The reaction was stopped after 5 minutes by adding 1.0 ml. of the iodine solution. The color was read at 620 m $\mu$  after the addition of 5.0 ml. of  $H_2O$ . When metabolic inhibitors or labeled amino acids were used, they were added to the flasks at the start of the incubation at 25 C.



Microscopy. All samples were either grown or germinated in a growth chamber with a 14 hour day and night temperatures of 23 and 17 C, respectively, except those shown in Figs. 1 and 4 which were germinated in moist sand in sterile petri dishes at room temperature.

Blocks 1-2 mm on the side were dissected from that part of the cotyledon distal to the axis attachment either before or after fixation. In the latter case only the seed coats were removed prior to fixation and the peas immersed as a whole.

The preparation of tissue for microscopy proceeded in five consecutive stages:

#### 1. Fixation.

A comparison of the results obtained after various types was found useful in order to reduce artifacts to a minimum.

Either one of three types was used, all at room temperature for 2-3 hours, with agitation:

- A. 2%  $\text{OsO}_4$  solution in distilled water
- B. 4%  $\text{KMnO}_4$  solution in 0.1 uranyl nitrate in distilled water
- C. An atmosphere saturated with water and  $\text{OsO}_4$  vapor.

#### 2. Dehydration.

Regardless of the type of fixation, all samples were dehydrated in a graded series of ethanol (50%, 70%, 100%).

### 3. Embedding.

Either one of two methods:

- A. In a 3:1 mixture of butyl and methyl methacrylate containing 1% Azo-di-iso-butyronitrile as catalyst. Polymerized at 60°C for 18 hrs.
- B. In epoxy resin according to the Araldite method of Luft (13). Polymerization was in three steps: at 30°C for 48 hours, followed by 40°C for 48 hours and terminated by 60°C for 24 hours.

This method results in excellent tissue preservation and eliminates much damage that was previously thought to occur at the fixation stage.

### 4. Sectioning.

All sectioning was done with glass knives on a Porter-Blum (Sorvall) microtome.

- A. For electron microscopy, sections are approx. 500-1000 Angstroms thick.
- B. For light microscopy, sections 0.5-1 micron thick are adequate for observation with phase optics.

### 5. Staining.

Sections of tissue fixed in  $\text{OsO}_4$  solution or vapor and embedded in epoxy resin were stained for both light and electron microscopy by floating them on either one of the following solutions for about 30 min. at room temperature:

A. 4%  $\text{KMnO}_4$  in 0.1 uranyl nitrate in distilled water.

B. A solution containing plumbite ions prepared according to Karnovsky (14).

The observations were carried out either with a Siemens Elmiskop Ia or a Zeiss-Opton model W microscope equipped with phase contrast optics.

### Results and Discussion

Structure and Function of Pea Cotyledon Cells. Because it had already been shown 1 - that the mitochondria of pea cotyledon cells gradually lose function during the first two weeks of the germination and growth of the seedling (1), 2 - that there is a rapid loss of protein from these cells during germination (15) and 3 - that there is a great increase in several enzymatic activities beginning on the third or fourth day of germination (2,7), we were interested in trying to correlate subcellular structure with these metabolic events. The first electron micrographs showed that a major part of the cell volume of mature cells is occupied by roughly spherical bodies about 2 microns in diameter with no visible internal structure (Fig. 1). These bodies have a limiting membrane (Figs. 2 and 2a), are readily visible in the light microscope (Fig. 3) and retain their identity (Fig. 4) during isolation as a centrifugal fraction

from an aqueous homogenate of the pea cotyledons. The main bulk of this fraction consists of relatively intact protein bodies (Fig. 4) obviously identical to the cytoplasmic bodies observed in whole cells (Figs. 1 and 3). Some contamination with cellular debris and artifacts resulting from the preparative procedure is apparent and expected because no attempt has been made to wash the pellet.

Analysis of the pellet and supernatant fractions by the extraction and precipitation procedure of Danielsson (11) indicate (Table I) that the pellet protein consists almost entirely of globulins. The small proportion of albumin in the pellet is probably accounted for by the fact that the pellet was not washed. It also appears that there is little globulin outside the protein bodies in solution in the cytoplasm because the globulins are soluble in the homogenizing medium. We have not studied extensively conditions for preserving the protein bodies in aqueous suspensions. They gradually go into solution when maintained in 0.1 M Tris (pH 7.1). However one has adequate time for the kind of separations and analyses reported here.

Figs. 5, 6 and 7 show the results of the chromatography of the various fractions on DEAE columns and further support the conclusion that the main component of the pellet and the protein bodies is the globulin fraction and that the globulin fraction is the major protein fraction of



the pea cotyledon cells. It is interesting to note that the chromatographic separation suggests the presence of four components in the globulin fraction while ultracentrifugal analyses indicate two major components (11).

The time of formation of the globulins and albumins during maturation of the peas and of their disappearance during germination has already been studied (17,15).

A preliminary examination of the development of the protein bodies during maturation of the pea (Figs. 9,10,11) indicates that the bodies become visible only at a relatively late stage in the maturation of the pea cotyledon. When these observations of the development of the protein bodies are compared with Danielsson's (17) chemical and physical analyses of peas at similar stages of development, it is apparent that most of the globulin synthesis may occur before the protein bodies are visible. If such a comparison of our data with Danielsson's is valid (in spite of differences in variety, environment, etc.), one can conclude that the globulins are synthesized, then formed into bodies in contrast to the possibility of having the protein bodies continuously increasing in size and/or number during the formation of the globulins.

During the early days of germination the protein bodies decrease in number, increase in diameter several fold and disappear (Figs. 12, 13, 14). A comparison with the

chemical and physical analyses of Danielsson (15) suggests that the protein bodies disappear before the breakdown of the globulin reserves. The loss of protein bodies and vacuolation of the cotyledon cells occurs first in the sub-epidermal cells and last in those cells at the center of the cotyledon.

It is apparent that a satisfactory elucidation of the role of these protein bodies in the protein economy of peas during maturation and germination will require a careful coordination of optical and electron microscopy with the appropriate chemical and physical analyses.

Because one of our theses is that the cotyledon cells of germinating peas are capable of doing synthetic work (1,2), we were pleased to find in our electron micrographs clearly defined endoplasmic reticulum (Figs. 1, 17), ribosomes (Figs. 2, 17), mitochondria (Figs. 15, 16), golgi bodies (not shown), nuclei and nucleoli. The presence of well-defined mitochondria in the dry cotyledons before germination was to be expected from the earlier observations that full respiratory capacity of the cotyledons is reached as soon as the cotyledon is fully imbibed. As germination progresses and the protein bodies and starch granules disappear and large vacuoles form, the mitochondria become a relatively prominent feature of the thin layer of cytoplasm remaining. Mitochondria are easily recognizable in thin

sections of cotyledons even after 16 days of germination (Fig. 16). Their poorly defined internal structure may be the cause of the decreased rate of respiration observed at this stage of germination (1).

The cotyledon cells also contain some well-defined chloroplast lamellae (Fig. 18) although they presumably have no function during germination. The cytoplasm of the cotyledon cells is interconnected by many plasmodesmata (Figs. 19 and 20). Presumably these intercellular bridges are of importance during the transport of the reserve materials to the growing parts of the plant.

The question of de novo synthesis vs. activation of pre-existing proteins as an explanation for the observed increases in enzymatic activities has been approached by the use of DEAE columns to separate the proteins of cotyledon extracts following incubation of the excised cotyledons in  $^{14}\text{C}$ -labeled amino acids. The supernatant fraction chromatographed in Fig. 8 was from cotyledons of three-day old germinating peas. The first pyrophosphatase peak may be an artifact. The three major peaks always appear during chromatography and the last pyrophosphatase peak increases greatly during the third to sixth days of germination. The small ATP-ase peak results from the low activity of the corresponding pyrophosphatase peak toward ATP at pH 7.1. The major ATP-ase peak increases greatly during germination and is

the enzyme studied earlier by Young and Varner (2). The proteins which become labeled in vivo from  $^{14}\text{C}$ -labeled amino acids (1) are in the albumin fraction and separate into five or six discrete peaks under the chromatographic conditions used here. One of these is associated with the pyrophosphatase peak which increases greatly during germination. Another coincides with the  $\text{Mg}^{++}$ -requiring phosphatase which increases during germination. Further characterization of these phosphatases and of the control of the timing of their appearance during germination is in progress.

#### The Control of $\alpha$ -amylase Formation by Gibberellic Acid.

Because preliminary experiments indicated that the formation of  $\alpha$ -amylase in peas was triggered by gibberellic acid, we decided to begin an intensive study of the control of  $\alpha$ -amylase formation. As experimental material we chose the barley endosperm system described by Paleg (8,9,10).

It seemed to us that the logical first step toward an understanding of the control of  $\alpha$ -amylase formation was to find out whether the increase in enzymatic activity resulted from an activation of some kind or from de novo synthesis. The inhibition of the formation of  $\alpha$ -amylase by dinitrophenol (Table II), anaerobiosis (Table II), p-fluorophenylalanine (Table III) and chloramphenicol (Table IV) is consistent with the possibility of de novo synthesis.



When the barley half-seeds were incubated with and without gibberellic acid in the presence of  $^{14}\text{C}$ -phenylalanine, the  $\alpha$ -amylase secreted into the buffer surrounding the gibberellic acid treated half-seeds appeared to be labeled (Figs. 21, 22). In fact, the radioactivity associated with the  $\alpha$ -amylase peak from the DEAE column constituted a major fraction of the total counts incorporated into protein. In the absence of gibberellic acid there are essentially no counts associated with the added carrier  $\alpha$ -amylase as it emerges from the DEAE column. Extraction of the gibberellic acid treated seed coats after incubation in  $^{14}\text{C}$ -phenylalanine shows that even in the cellular extract the radioactivity associated with  $\alpha$ -amylase is an appreciable fraction of the total radioactivity incorporated into soluble proteins (Fig. 23). We consider these experiments with labeled phenylalanine as further evidence consistent with the idea that in barley,  $\alpha$ -amylase is formed by de novo synthesis. Proof that the  $\alpha$ -amylase arises by de novo synthesis requires purification of the labeled  $\alpha$ -amylase to a state of homogeneity and demonstration that the labeled phenylalanine is distributed uniformly along the polypeptide chain.

The capacity for formation of  $\alpha$ -amylase by barley half-seeds is localized in the aleurone cells (Table V). The presence of the starchy endosperm appears to decrease the formation of  $\alpha$ -amylase in the absence of added gibberellic

acid and to aid the release of  $\alpha$ -amylase in the presence of added gibberellic acid.

We believe that the barley half-seeds not only provide a good system for the study of enzyme formation in mature (senescent) cells but that it is also an excellent system for the study at the molecular level of the mechanism of action of gibberellic acid.

#### Acknowledgment

We gratefully acknowledge the technical assistance of Miss Nancy Joseph.

## Bibliography

1. Young, J. L., Ru Chih Huang, S. Vanecko, Joy D. Marks, and J. E. Varner. 1960. Conditions affecting enzyme synthesis in cotyledons of germinating seeds. *Plant Physiol.* 35:288.
2. Young, J. L., and J. E. Varner. 1959. Enzyme synthesis in the cotyledons of germinating seeds. *Archiv. Biochem. Biophys.* 84:71.
3. Varner, J. E., and G. Schidlovsky. 1963. Intracellular distribution of proteins in peas. *Plant Physiol.* In press.
4. Dieckert, J. W., and J. E. Snowden, Jr. 1960. Sub-cellular distribution of substances in seeds of *Arachis Hypogaea*. *Fed. Proc.* 19:126.
5. Altschul, A. M., J. E. Snowden, Jr., D. D. Manchon, Jr., and J. M. Dechary. 1961. Intracellular distribution of seed proteins. *Archiv. Biochem. Biophys.* 95:402.
6. Bagley, B. W., J. H. Cherry, Mary T. Rollins, and A. M. Altschul. 1963. A study of protein bodies during germination of peanut seed. *Am. J. Botany.* In press.
7. Varner, J. E., L. V. Balce and R. C. Huang. 1963. Senescence of cotyledons of germinating peas. Influence of axis tissue. *Plant Physiol.* In press.
8. Paleg, L. 1960. Physiological effects of gibberellic acid. I. On carbohydrate metabolism & amylase activity of barley endosperm. *Plant Physiol.* 35:293.

Bibliography (Cont.)

9. Paleg, L. 1960. Physiological effects of gibberellic acid. II. On starch hydrolyzing enzymes of barley endosperm. *Plant Physiol.* 35:902.
10. Paleg, L. 1961. Physiological effects of gibberellic acid. III. Observations on its mode of action on barley endosperm. *Plant Physiol.* 36:829.
11. Danielsson, C. E., and H. Lis. 1952. Differences in the chemical composition of some pea proteins.
12. Shuster, L., and R. H. Gifford. 1962. Changes in 3'-nucleotidase during the germination of wheat embryos. *Archiv. Biochem. Biophys.* 96:534.
13. Luft, J. H. 1961. Improvements in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.* 9:409.
14. Karnovsky, M. M. 1961. Simple methods for "staining with lead" at high pH in electron microscopy. *J. Biophys. Biochem. Cytol.* 11:729.
15. Danielsson, C. E. 1951. The breakdown of the high molecular reserve proteins of peas during germination. *Acta Chemica Scandinavica.* 541.
16. Whaley, G. W., H. H. Mollenhauer and J. H. Leech. 1960. The ultrastructure of the meristematic cell. *Amer. J. Bot.* 47 (6):401. 1960.
17. Danielsson, C. E. 1952. A contribution to the study of the synthesis of the reserve proteins in ripening pea seeds. *Acta Chemica Scandinavica.* 149.



18. Schidlovsky, G., Structure of the photochemical apparatus in photosynthesis, Technical Documentary Report No. AMRL-TDR-62-116, October 1962, 6570th Aerospace Medical Research Laboratories, Wright-Patterson Air Force Base, Ohio.

### Abbreviations Used in Figures

PB	protein body
ER	endoplasmic reticulum
ES	ergastic substance
M	mitochondrion
S	starch
W	cell wall
PM	plasma membrane
PD	plasmodesmata
V	vacuole
E	limiting membrane of protein body
CY	cytoplasm
N	nucleus
L	chloroplast lamellae
G	granum
ML	middle lamella

Table I  
Distribution of Globulins and Albumins  
in Homogenate of Pea Cotyledons

	ALBUMINS	GLOBULINS
SUPERNATANT FRACTION	120 mg.	22 mg.
PELLET	20	150
DEBRIS	<u>56</u>	<u>180</u>
TOTAL	196	352
0.2 M NaCl EXTRACT OF GROUND COTYLEDONS	165	410

The albumins and globulins of each fraction were determined by the biuret procedure after separation according to Danielsson (11).

Table II

The Effects of Gibberellic Acid, Dinitrophenol  
and Anaerobiosis on the Development of  $\alpha$ -amylase

TREATMENT	$\alpha$ - AMYLASE IN	
	RINSE	EXTRACT
H <sub>2</sub> O	0.00	0.06
GA, 10 <sup>-6</sup> M	0.97	0.76
DNP, 10 <sup>-3</sup> M	0.00	0.10
DNP, 10 <sup>-4</sup> M	0.04	0.15
DNP, 10 <sup>-5</sup> M	0.30	0.31
GA, 10 <sup>-6</sup> M ANAEROBIC	0.07	0.10

The numbers indicate the changes in O.D. units of the starch-iodine complex and are directly proportional to the amount of  $\alpha$ -amylase present.



Table III

The Effect of  $p$ -fluorophenylalanine  
on the Development of  $\alpha$ -amylase

TREATMENT	$\alpha$ - AMYLASE IN	
	RINSE	EXTRACT
H <sub>2</sub> O	0.07	0.17
GA, 10 <sup>-6</sup> M	0.37	0.37
pF $\phi$ ALA, 10 <sup>-4</sup> M	0.11	0.12
" 10 <sup>-5</sup>	0.13	0.22
" 10 <sup>-6</sup>	0.44	0.40

The numbers indicate the changes on O.D. units of the starch-iodine complex and are directly proportional to the amount of  $\alpha$ -amylase present.

Table IV

The Effect of Chloroamphenicol on the  
Development of  $\alpha$ -amylase

	$\alpha$ -AMYLASE	
	RINSE	EXTRACT
H <sub>2</sub> O	0.10	0.14
GA, 10 <sup>-6</sup> M	0.44	0.76
GA + CHLORAMPHENICOL	0.06	0.54

The numbers indicate the changes in O.D. units of the starch-iodine complex and are directly proportional to the amount of  $\alpha$ -amylase present. The chloroamphenicol concentration was 1 mg./ml.

Table V  
The Source of the  $\alpha$ -amylase

	H <sub>2</sub> O		GA	
	RINSE	EXTRACT	RINSE	EXTRACT
COATS	0.13	0.30	0.23	0.80
STARCHY ENDOSPERM	0.09	0.05	0.09	0.02
COAT + ENDOSPERM	0.16	0.05	0.51	0.51

The numbers indicate the changes in O.D. units of the starch-iodine complex and are directly proportional to the amount of enzyme present. The "coats" fraction is prepared by squeezing the starchy endosperm out of the pre-incubated half-seed. The coat includes the aleurone layers and the outer layers of dead cells. The "coat + endosperm" fraction is comprised of dissected coats mixed with dissected starchy endosperm.

## Figure 1

Electron micrograph of pea cotyledon cells close to epidermis. Whole pea germinated for 18 hours on moist sand in sterile petri dish at room temperature.  $\text{KMnO}_4$ -Uranyl nitrate fixation. Epoxy resin embedding. No staining.

A large part of the cell volume is occupied by the protein bodies (PB) which after these preparatory methods appear less opaque than the surrounding cytoplasmic matrix. Their distribution throughout the cytoplasm is even and they do not appear to be associated with any particular cytoplasmic organelle. The irregularity of their outline is believed to be at least partly due to water imbibition prior to fixation and to an artifact arising during fixation. Figures 2 and 12 suggest that they are nearly spherical when in the dry state.

Agglomerations of endoplasmic reticulum (ER) are seen at the periphery and the interior of the cell. Mitochondria and Golgi bodies are present in these cells although not clearly visible at this magnification (see fig. 15). Some plasmodesmata (PD) and starch granules (S) are seen.

A morphologically distinct component, staining densely under the above conditions, and designated here by ES (ergastic substance) has been consistently recognized (after all fixation methods used here) in mature as well as



Figure 1 (Cont.)

germinating pea cotyledon cells. At early stages of germination, it is most prominent in areas adjacent to the plasma membrane, and at the periphery of starch granules and chloroplasts. Its function and chemical nature are unknown. After 6 days of germination this substance is no longer identifiable by the methods used here.

Magnification: 9,500 x







Figures 2 and 2a

Electron micrograph of a portion of a pea cotyledon cell. Non-germinated whole dry pea fixed in  $\text{OsO}_4$  vapors. Epoxy embedding. Lead stain (13).

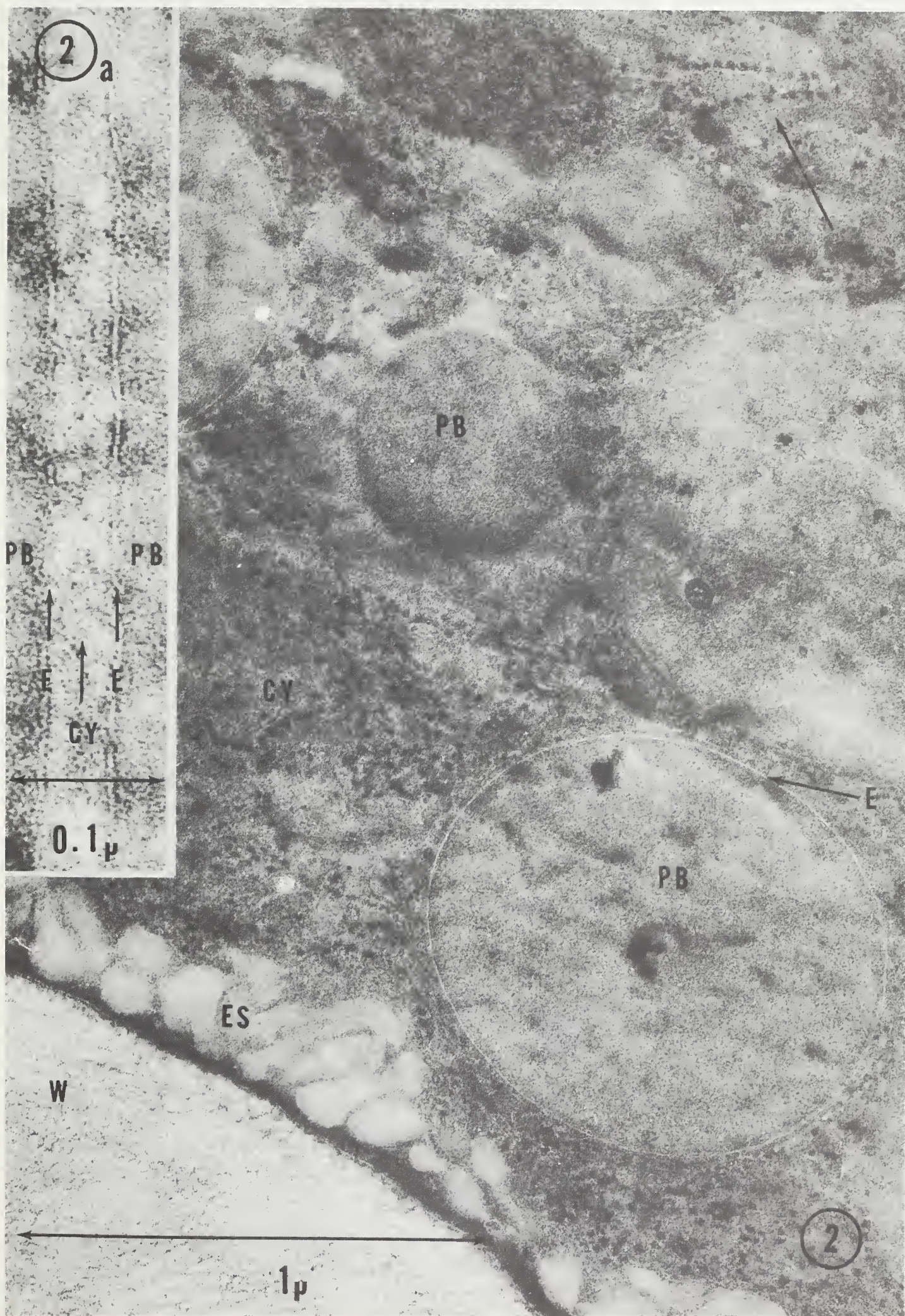
The protein bodies appear nearly spherical and distinctly delineated by a membrane. The latter is approx. 75 Angstroms thick and is most prominent here because of its clear central band. Figure 2a shows this membrane to be of the "unit membrane" type (16), i.e. a triple-layered structure with two dark bands separated by a clear band each approx. 25 Angstroms thick. The same structure is found in the plasma membrane (figs. 19 and 20). The substance ES is now less opaque than the background, but still a distinct morphological entity. The cytoplasm (CY) is filled with dense particles reminiscent of microsomes. The structure at the unmarked arrow suggests that these particles may be associated with portions of the endoplasmic reticulum. The membrane of the protein body in the center of the picture is not seen because of its unfavorable orientation with respect to the plane of sectioning. The opaque deposits on the lower PB and above it are contamination of lead carbonate arising during staining.

Fig. 2a shows the membranes (E) of two protein bodies (PB) which are separated by a narrow strand of cytoplasm (CY).

Fig. 2: 78,000 x

Fig. 2a: 257,000 x







### Figure 3

A light micrograph of pea cotyledon cells. Fresh pea, 21 days from flowering, 10 mm diameter. Fixation in  $\text{OsO}_4$  solution. Epoxy embedding. Potassium permanganate-uranyl nitrate staining.

Illustrates various appearances of protein bodies ( $\text{PB}_1$  -  $\text{PB}_2$  -  $\text{PB}_3$ ) frequently encountered in late maturation and early germination samples. This variation is attributed to a fixation artifact. Its occurrence raises serious difficulties in the morphological analysis of the processes of formation and disappearance of protein bodies at the electron microscope level.

### Figure 4

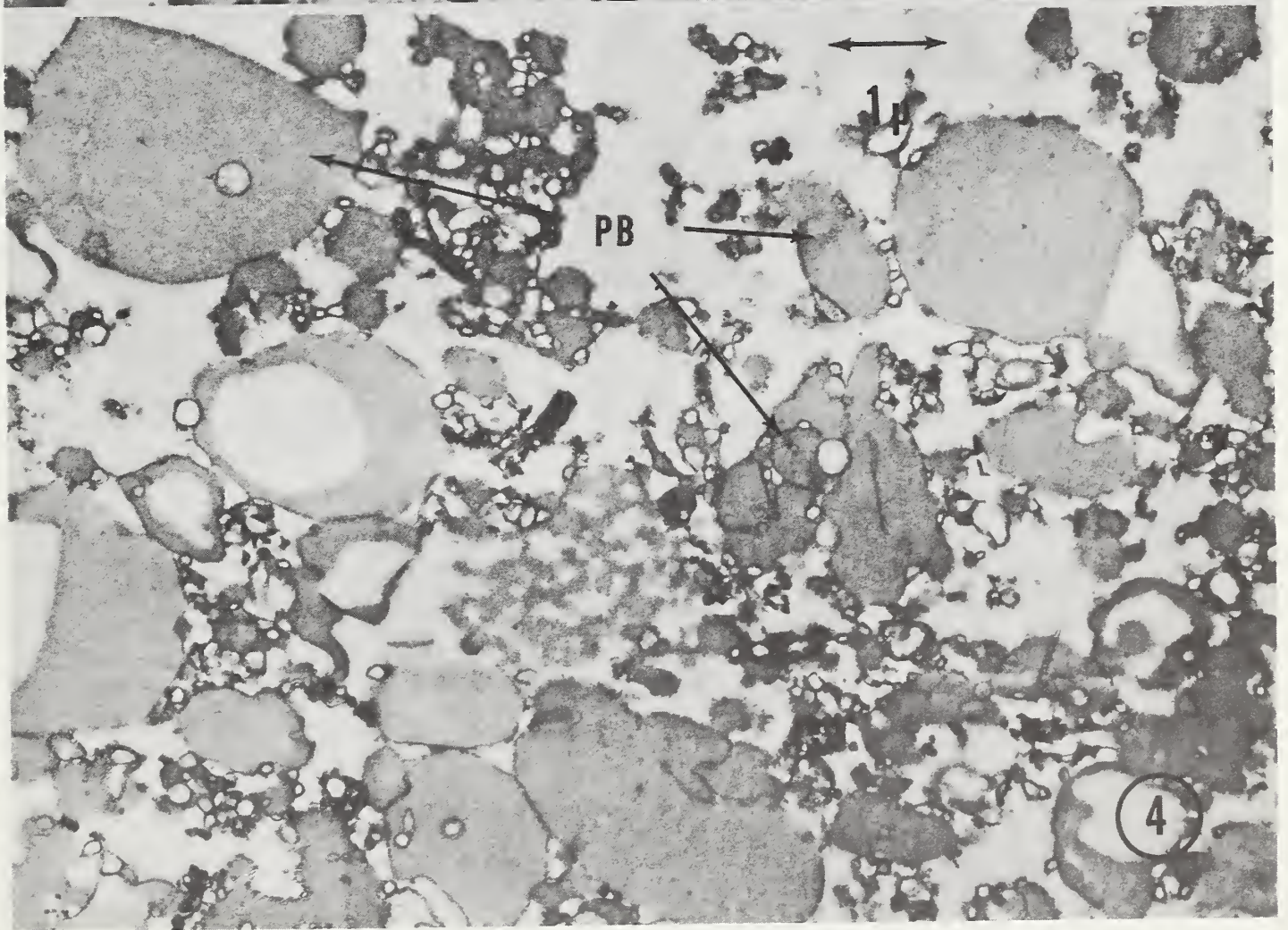
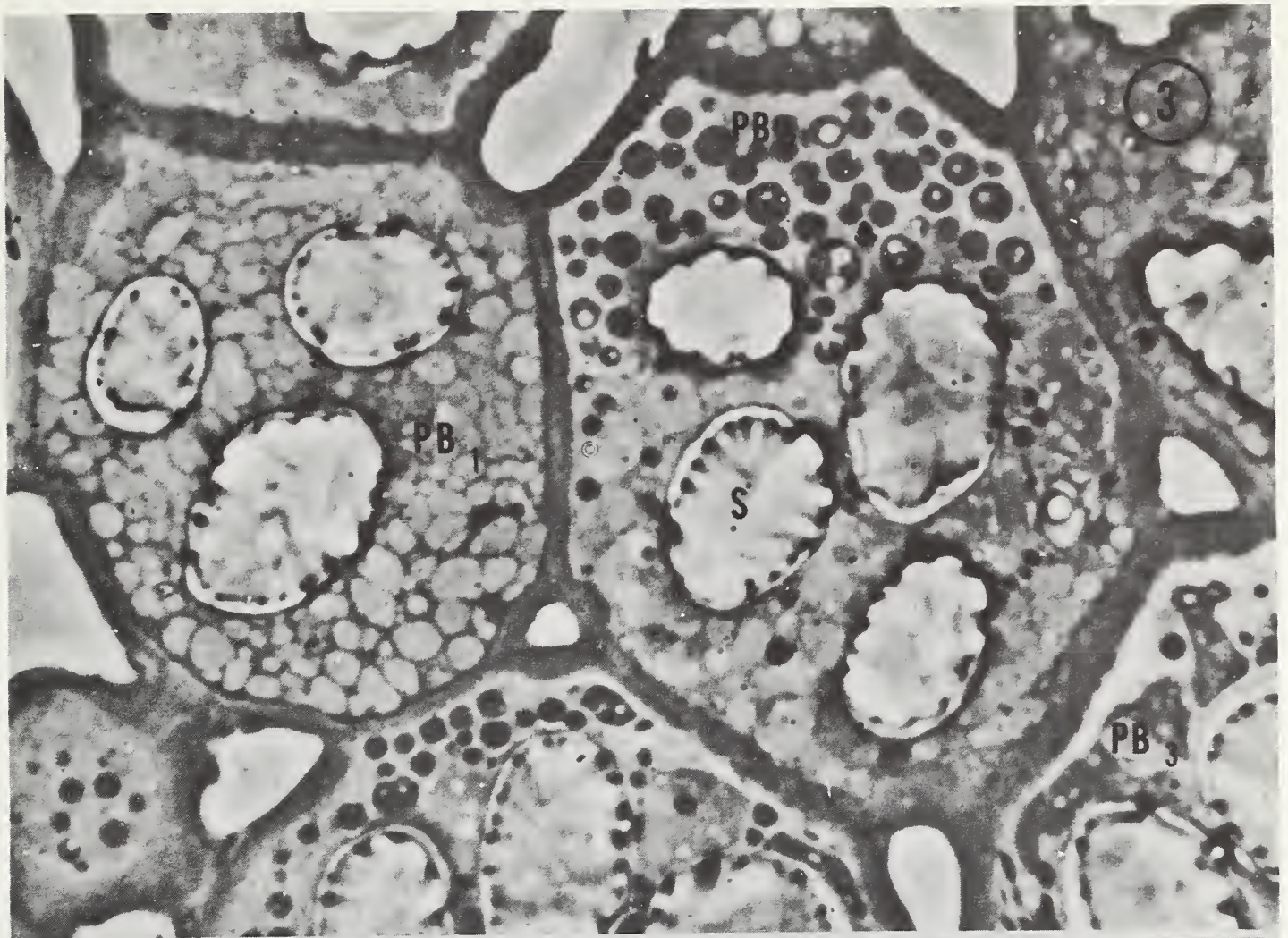
Electron micrograph of a section through a pellet of the "globulin" fraction.

Potassium permanganate-uranyl nitrate fixation. Methacrylate embedding. No staining.

The pellet consists mainly of protein bodies (PB) in various states of preservation. Some of them appear vacuolated, as they also do sometimes in situ (see  $\text{PB}_2$  in Fig. 3). Some contamination in the form of small vesicles and dense irregular shapes is present because no attempt has been made to purify the pellet.

Magnification: 13,200 x







## Figures 5 to 8

DEAE - cellulose chromatograms of various cotyledon materials. The preparation of the materials is described in Methods. In each case, elution was accomplished by a gradient of NaCl (in 0.01 M Tris, pH 7.8) increasing linearly from 0 to 0.5 M. Fractions were 6 ml. each. The column was 1.1 x 20 cm.

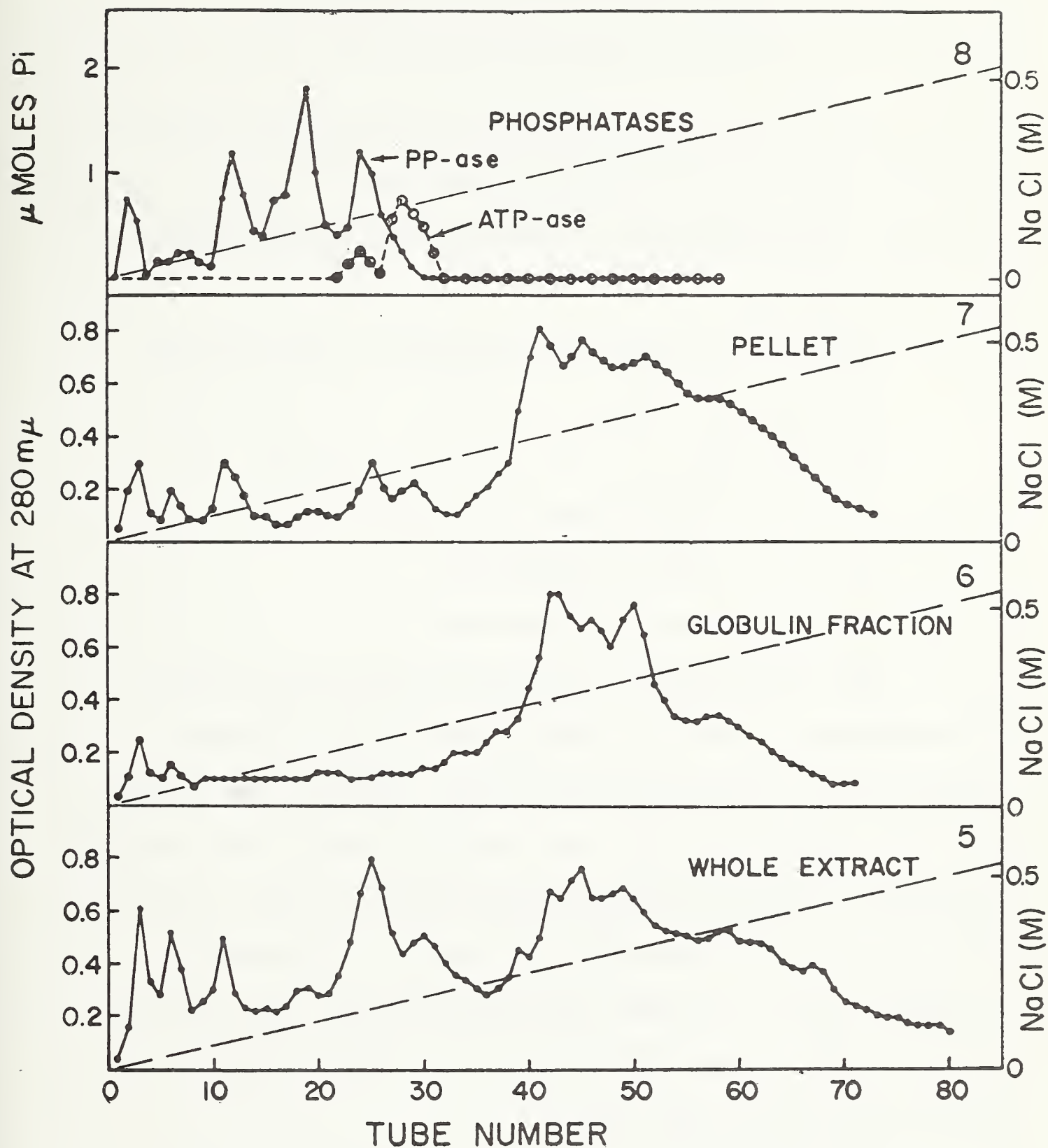
In Figures 5, 6 and 7 protein concentration was followed by measuring optical density at 280 m $\mu$ . Figure 8 represents adenosine triphosphatase (ATP-ase) and pyrophosphatase (PP-ase) activity in the various fractions. Enzymic activity was measured as  $\mu$  moles of Pi formed from potassium pyrophosphate and ATP as described under Methods.

Figure 5 - Whole extract obtained from 24 hour germinated peas.

Figure 6 - Globulin fraction from 24 hour germinated peas.

Figure 7 - Pellet fraction from 24 hour germinated peas.

Figure 8 - Supernatant fraction (144,000 x g ) from peas germinated for 72 hours.





Figures 9, 10 and 11 - Maturation

Figures 12, 13 and 14 - Germination

Phase contrast micrographs of pea cotyledon cells at various stages of maturation and germination. Fixed in  $\text{OsO}_4$  solution. Epoxy embedding. Potassium permanganate-uranyl nitrate staining.

Fig. 9 - 13 days after flowering - 6 mm diameter

Fig. 10 - 16 " " " - 8.5 mm "

Fig. 11 - 21 " " " - 10 mm "

Fig. 12 - Dry, mature, before germination

Fig. 13 - 4 days' germination

Fig. 14 - 7 days' germination

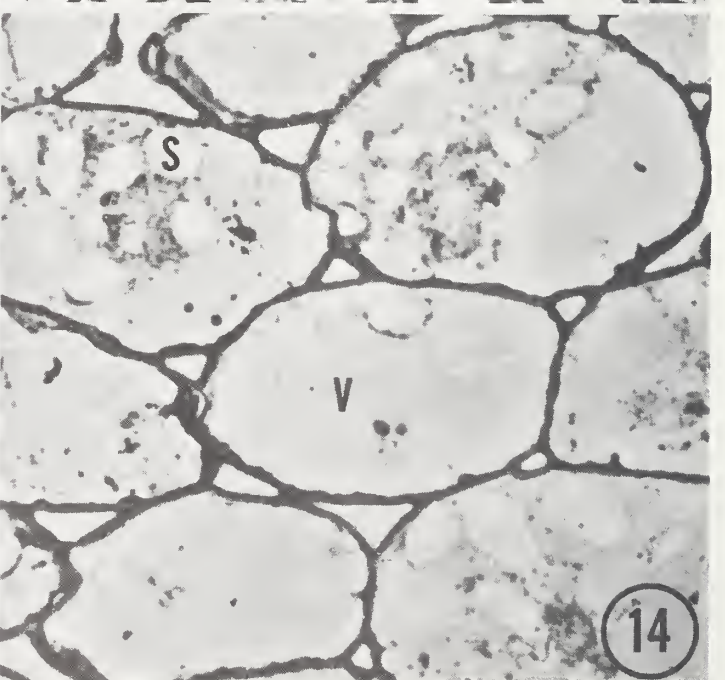
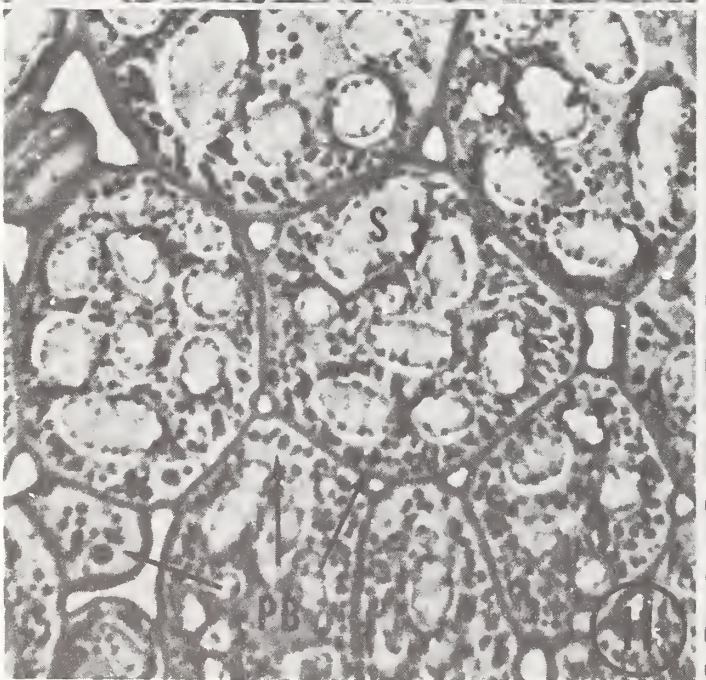
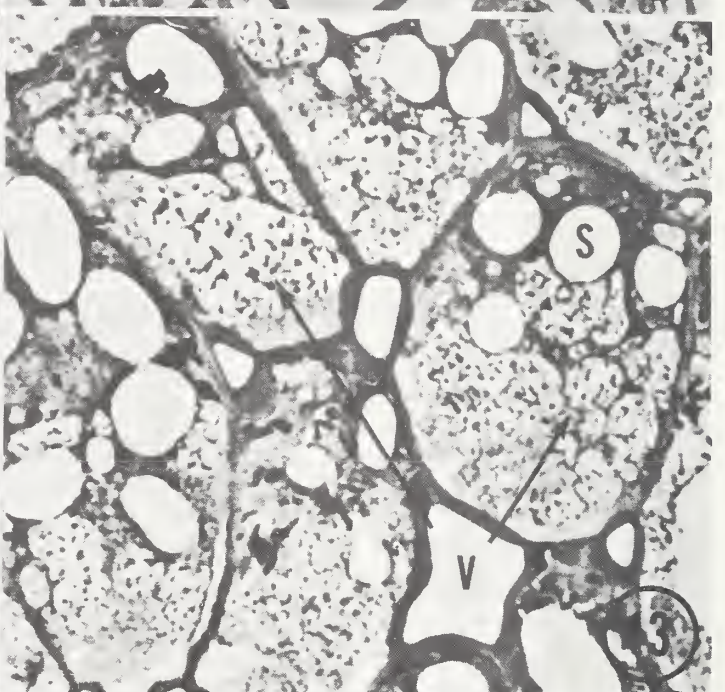
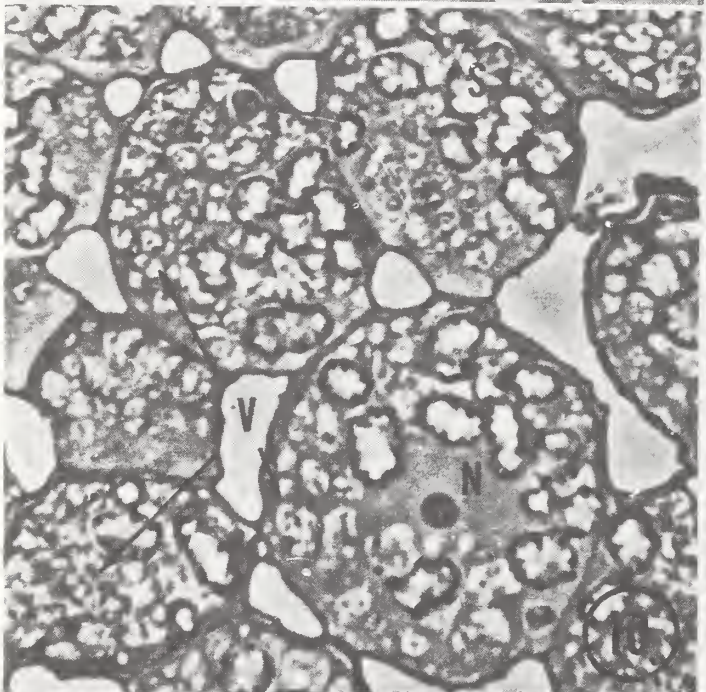
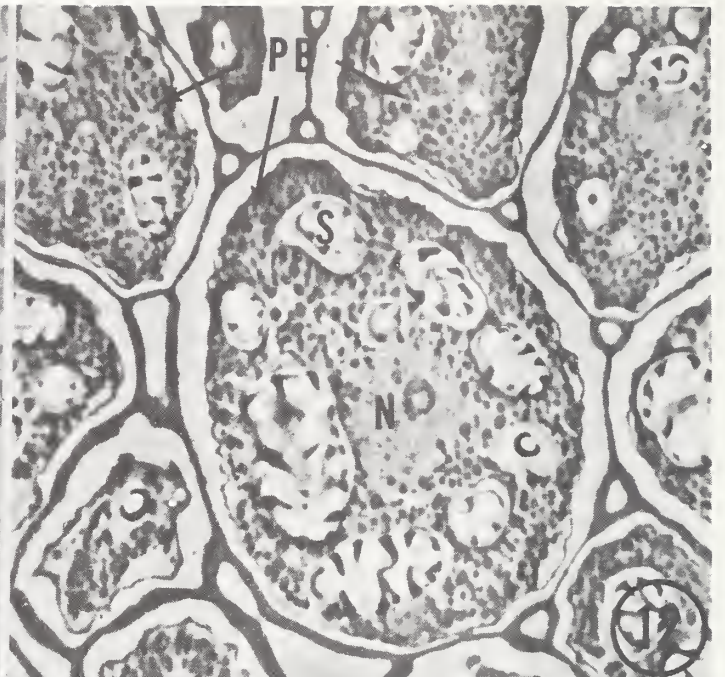
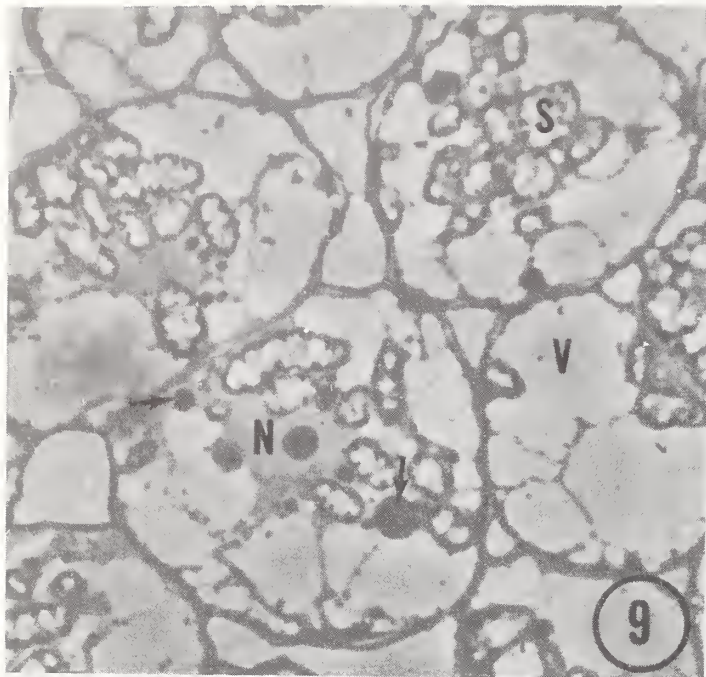
The morphology of the processes of formation and disappearance of protein bodies appears to be strikingly similar at the light microscope level. They both go through a stage at which the cells are filled with many small vacuoles containing irregularly shaped dense deposits (Figs. 10 and 13). As maturation proceeds, the vacuoles disappear (or perhaps increase in number and become filled). The protein bodies become recognizable as such only during the very last days of maturation (Fig. 11).

During germination, the protein bodies lose their identity by the 4th day, and are gradually replaced by a decreasing number of vesicles. By the 7th day the cells consist of only a few large vacuoles and a thin sheet of cytoplasm at the periphery of the cells (Fig. 14). Their

general appearance is then comparable to that of young cells in the maturation series (Fig. 9).

The unmarked arrows on Fig. 9 emphasize some unidentified regularly occurring bodies in young cells.







## Figures 15 and 16

Electron micrographs of mitochondria (M) in pea cotyledon cells. Fixed in  $\text{OsO}_4$  solution. Epoxy embedding. Potassium permanganate-uranyl nitrate staining.

Figure 15 - Mature, dry pea, from germination

Figure 16 - 16 days' germination.

Before the onset of germination, mitochondria appear filled with regularly oriented cristae, a condition rarely seen in plant cells. By the 16th day of germination, the cristae are disorganized and probably fewer in number, but the mitochondrion still retains its identity.

Magnification: 70,000 x

## Figure 17

Portion of a pea cotyledon cell after 3 days of germination. Fixed in  $\text{OsO}_4$  solution. Epoxy embedding. Potassium permanganate-uranyl nitrate staining.

At this stage there is a proliferation of endoplasmic reticulum (ER). It appears to be associated with particles and is therefore of the "rough surface" type. The ergastic substance (ES) appears to have decreased in amount and to be moving toward the interior of the cell.

Magnification: 30,000 x

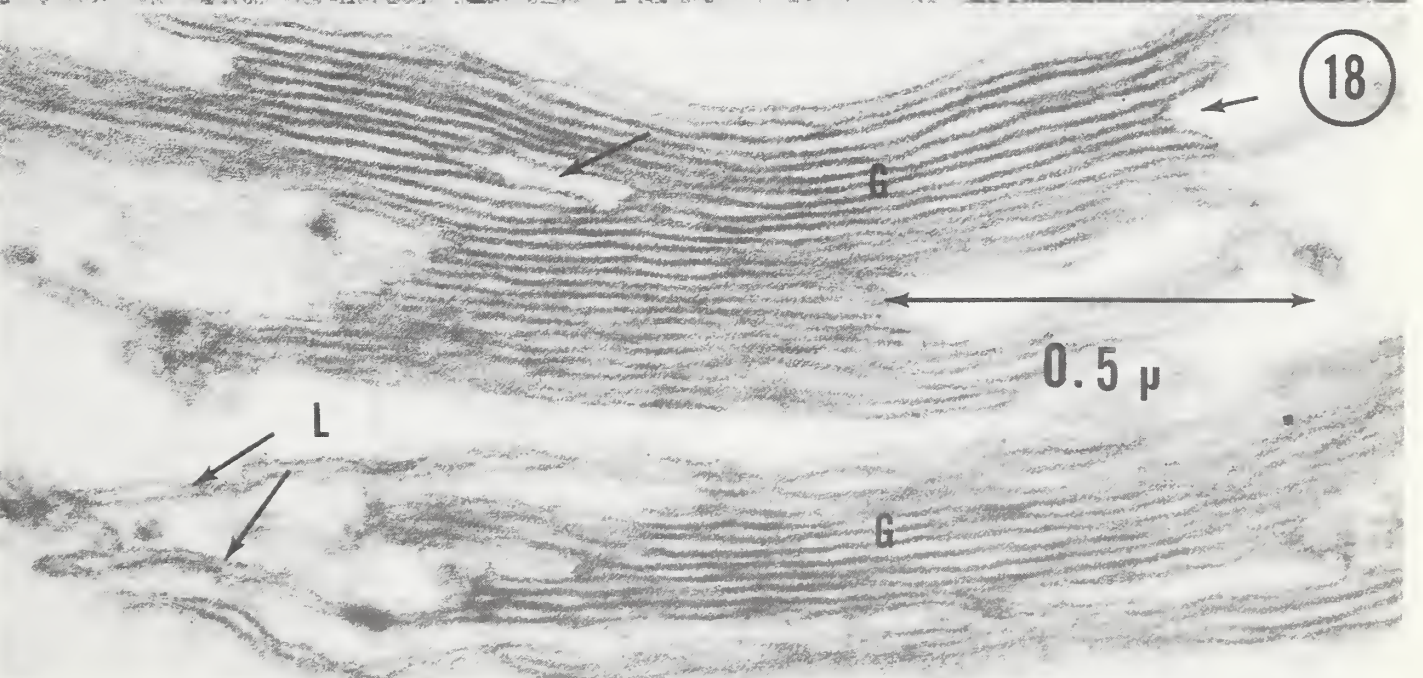
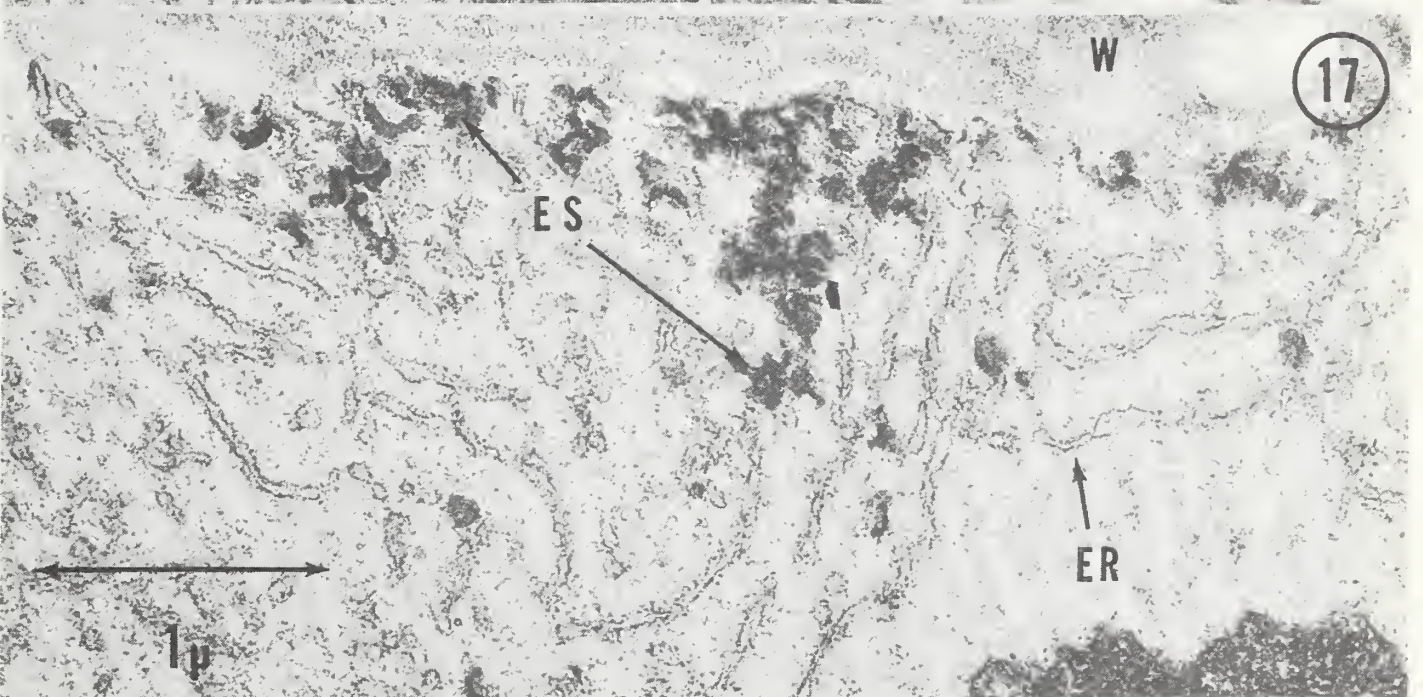
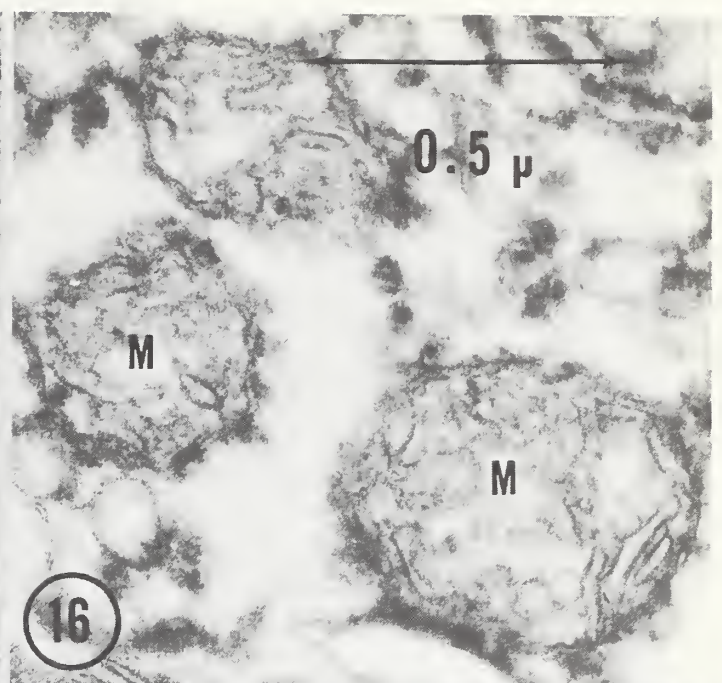
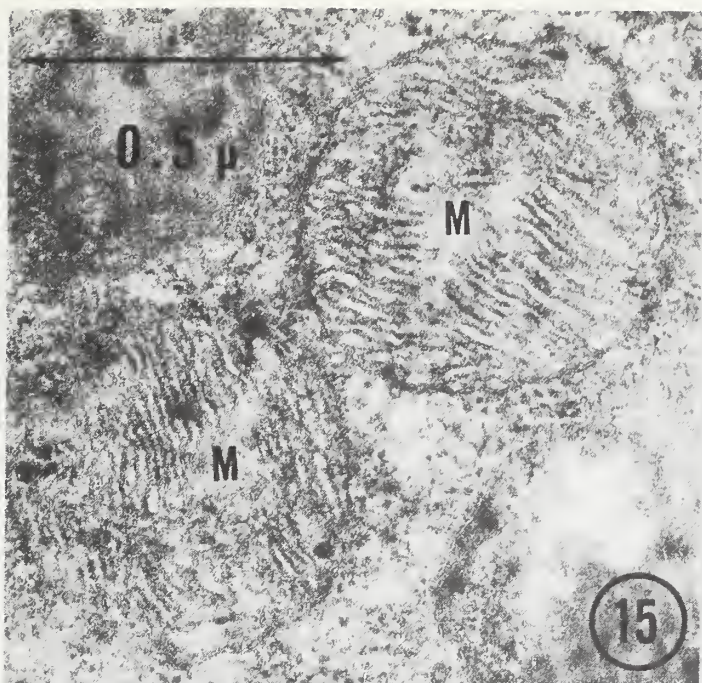


### Figure 18

Same sample as described in Fig. 17. The electron micrograph shows chloroplast lamellae in close proximity to a starch granule. The lamellae (L), which have been described as "closed flattened sacs," are seen here to form grana (G) by close apposition to each other as described previously in higher plant and algal chloroplasts (18). The unmarked arrows point to areas in grana where the ends of lamellae and their manner of apposition is clearly seen.

Magnification: 92,000 x







## Figures 19 and 20

Electron micrographs of intercellular connections (plasmodesmata, PD) in pea cotyledon tissue, after 16 days of germination. Fixed in  $\text{OsO}_4$  solution. Epoxy embedding. Potassium permanganate-uranyl nitrate staining.

Four plasmodesmata at different orientations with respect to the plane of sectioning are shown in Fig. 19. A correlation of such appearances indicates that the plasmodesmatic channels are not simple rectilinear tubes. Their structure in the central region of the wall is somehow influenced by the presence of the middle lamella (ML). However, they suggest that the cytoplasmic matrix of neighboring cells may be continuous.

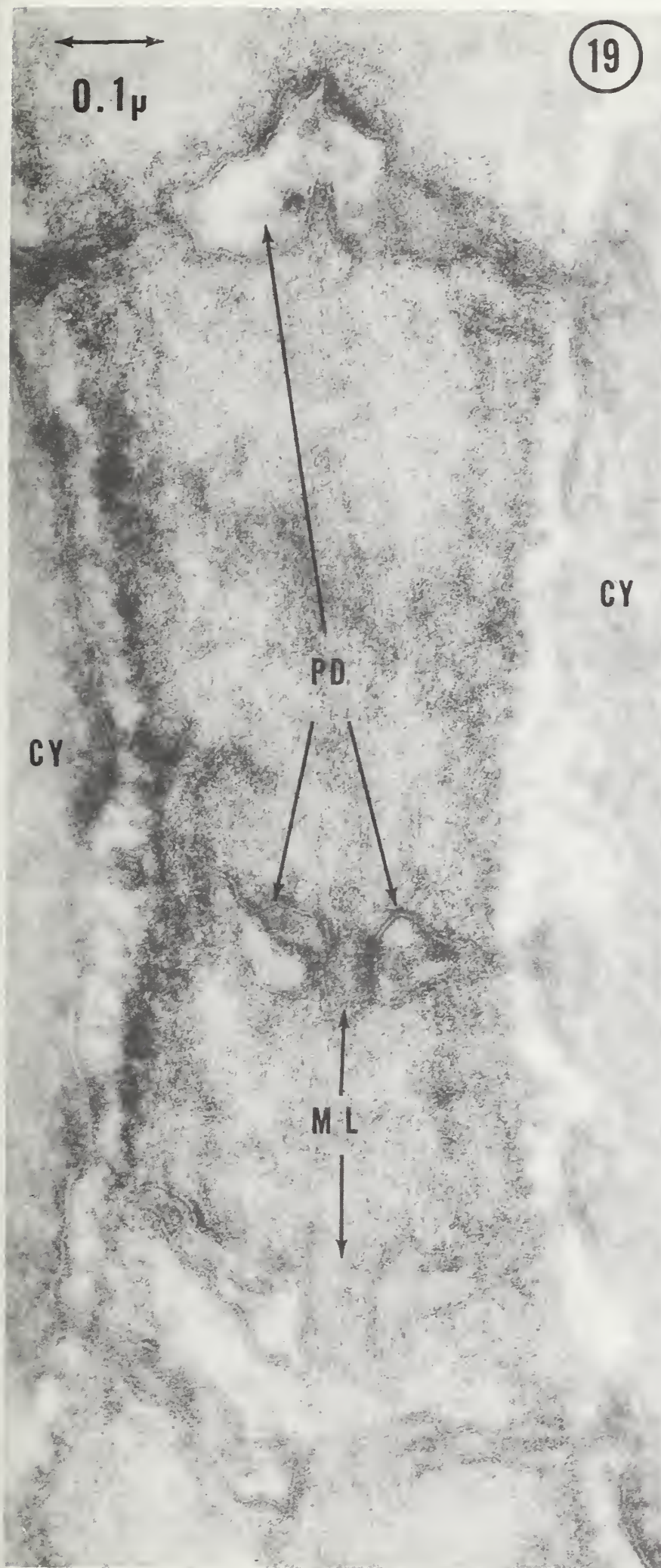
As seen in Fig. 20 the cytoplasmic projection into the wall is a cylinder of approximately 500 Angstroms' diameter. This diameter increases toward the middle lamella. The triple-layered membrane outlining the channels through the cell wall is obviously a direct extension of the plasma membrane as seen at PM on fig. 20.

It is not known whether the number and shape of such connections changes with metabolic activity.

Magnification: Fig. 19: 136,000 x

Fig. 20: 147,000 x







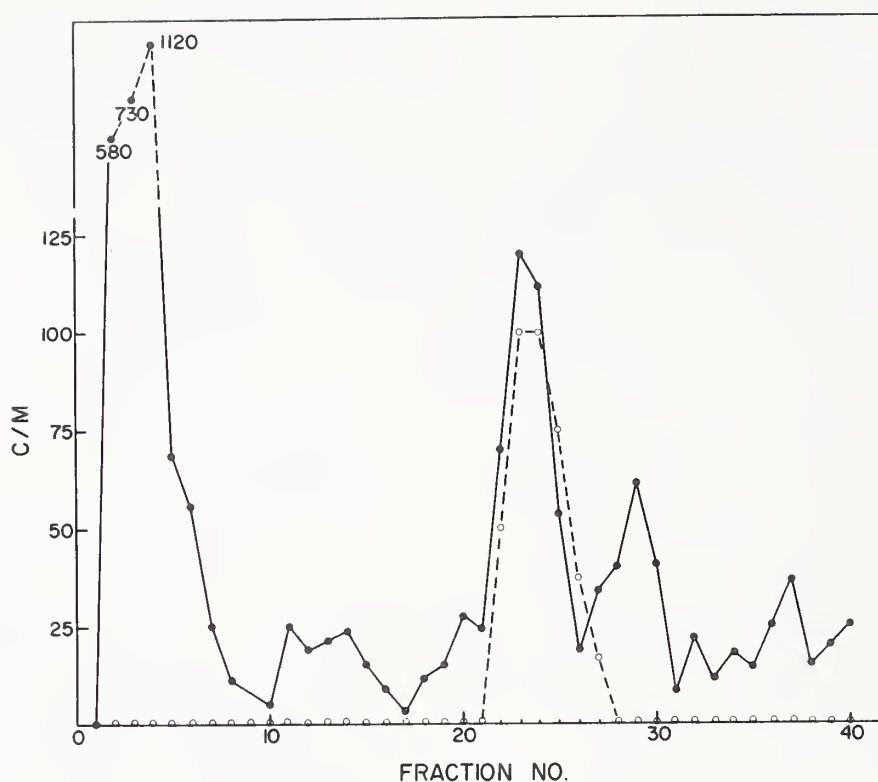


Figure 21

Distribution of counts after column separation on DEAE of the proteins released by 30 pre-incubated barley half-seeds incubated 24 hours at 25 C in 25  $\mu$ c of  $^{14}$ C-phenylalanine and  $10^{-6}$  M gibberellic acid in a final volume of 3.0 ml. The sample was dialyzed against several changes of a solution containing 0.001 M  $\text{Ca}^{++}$ , 0.015 M Tris (pH 7.8) and 0.001 M phenylalanine. The DEAE column was 1.2 x 20 cm. It was developed by application of a gradient of sodium chloride (in 0.001 M  $\text{Ca}^{++}$ , 0.015 M Tris (pH 7.8)) increasing linearly from 0 to 0.5 M. Each fraction contained 10 ml. Aliquots of 1.0 ml. were taken for counting on a gas flow

counter (about 35% efficiency). Aliquots of 0.2 ml. were taken for  $\alpha$ -amylase assay. The solid circles represent radioactivity while the open circles represent  $\alpha$ -amylase activity. There was essentially complete recovery of both the radioactivity and the  $\alpha$ -amylase activity added to the column.

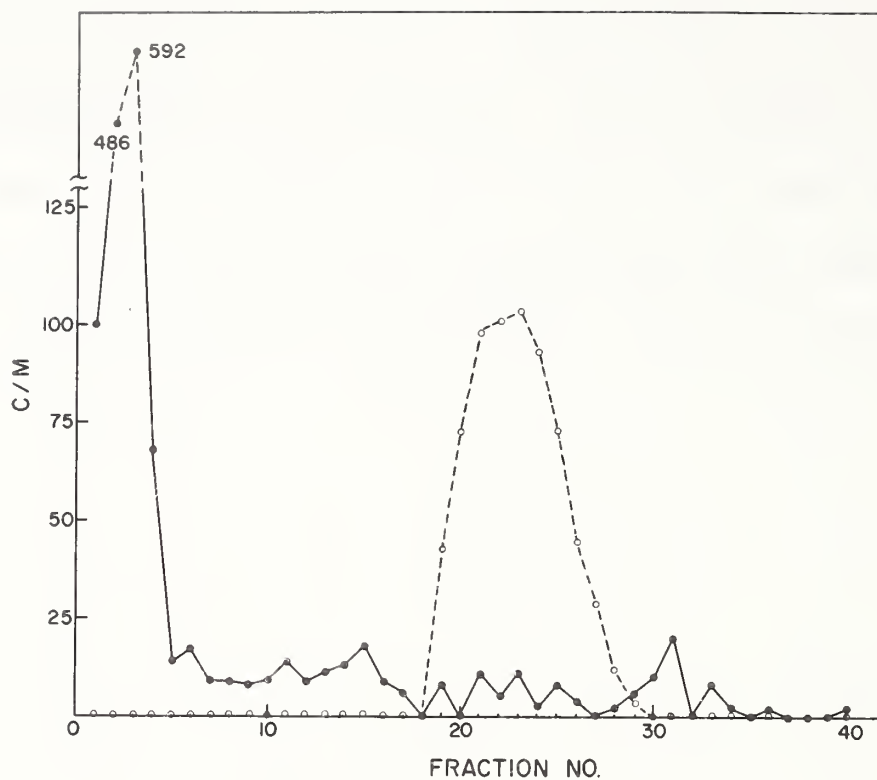


Figure 22

Distribution of counts after column separation on DEAE of the proteins released by 30 pre-incubated barley half-seeds incubated 24 hours at 25 C in 25  $\mu$ c  $^{14}$ C-phenyl-alanine in 3.0 ml. of water without added gibberellic acid. Other details were as described in the legend of Fig. 21.

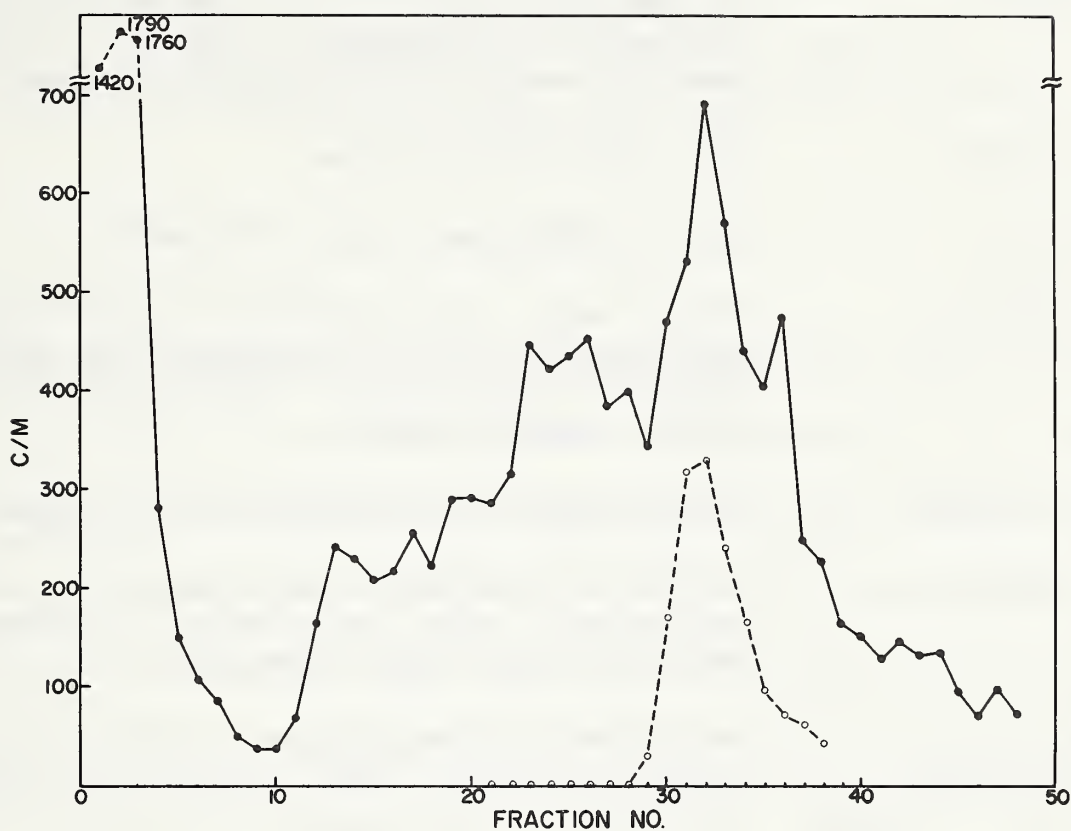


Figure 23

Distribution of counts after column separation on DEAE of the proteins extracted (by grinding in a mortar with 0.015 M Tris (pH 7.8)) from 20 seed coats (obtained from pre-incubated half-seeds) incubated 24 hours at 25 C in 25  $\mu$ c of  $^{14}\text{C}$ -phenylalanine and  $10^{-6}\text{M}$  gibberellic acid in a final volume of 3.0 ml. All other details as in Fig. 21 except fraction size was 8 ml.



## Discussion

- Dr. Abraham Marcus: Is there any loss of starch when barley is incubated with gibberillic acid? Is there any loss of starch when  $\alpha$ -amylase is formed?
- Dr. Joseph E. Varner: No. (To both questions.)
- Dr. Abraham Marcus: How were the pea slices labeled?
- Dr. Joseph E. Varner: In two ways; (1) by addition of  $C^{14}$  during germination, and (2) by dipping the cotyledon slices into the label. The results of the two methods were the same.
- Dr. Lawrence Y. Yatsu: Do you always see mitochondria in seeds?
- Dr. Joseph E. Varner: We have not looked at many kinds of seeds, particularly under the electron microscope, only under the light microscope.
- Dr. Max Milner:  
(To any of the morning speakers) Are there any observations on the originating activity (minutes after germination starts)?
- Dr. Joseph E. Varner: It. is usually about 18 hours before full rate  $O_2$  uptake is reached. If pea seeds are cracked into several pieces with a hammer, the same  $O_2$  uptake is reached in 2 hours.

## LYSOSOME-LIKE BEHAVIOR OF ACID PHOSPHATASE IN GERMINATING ONION SEEDS

James F. Harrington and Aaron M. Altschul  
Southern Utilization Research and Development Division  
New Orleans, Louisiana

### ABSTRACT

There is considerable evidence that particles, called lysosomes, containing several hydrolyzing enzymes, are present in liver and other animal tissues. To determine if lysosomes are also present in plant cells, germinating onion seeds were studied. Acid phosphatase activity was measured in a particulate fraction between 800 g (10 min.) and 20,000 g (10 min.) resuspended in 0.25 molar sucrose buffered at pH 5.0, with beta-glycerol-phosphate as the substrate. Aliquots with and without 0.1 percent Triton X-100 were compared. The difference was considered to be activity of acid phosphatase bound in lysosomes. No activity was found in dry seed but, with germination at 30°C, activity increased rapidly to the third day and then remained essentially constant. The activity was almost entirely in the embryo tissue. The peak activity in different experiments ranged from 204 to 267  $\mu$ g of phosphorous released per mg. of protein, and 73 to 89% of the activity was bound activity. It is concluded from these data that there is strong biochemical evidence that lysosome-like particles are present in embryos of germinating onion seed.

## Discussion

- Dr. Abraham Marcus: How do you know these aren't mitochondria?
- Dr. James F. Harrington: Mitochondria contain a different group of enzymes; also they differ in size.
- Dr. Te May Ching: Germinating peas, barley and Douglas fir seeds also showed lysosomal acid phosphatase activity; however, it was very low. In animal materials, lysosomes can be induced by X-ray irradiation, and probably are formed from mitochondria and other cytoplasmic inclusions when normal metabolism is disturbed by external factors.
- Dr. O. Z. Sellinger: I think you should test some other nonhydrolytic enzyme to prove that the hydrolase, phosphatase is lysosomal and not mitochondrial.

# PROTEIN IN THE DEVELOPING SOYBEAN COTYLEDON

Robert W. Howell  
U. S. Regional Soybean Laboratory  
Crops Research Division  
Urbana, Illinois

## ABSTRACT

Protein and other nitrogen fractions of developing seeds are in a dynamic state, reflecting changes which are related to the age of the seed and presumably to environmental factors. In very young soybean seeds, only about half of the nitrogen is in protein, but the protein increases steadily and usually includes about 95% of the total nitrogen at maturity. The proportion of nucleic acids is relatively constant on a weight basis, but the soluble nucleotides show a decrease during seed development comparable to that of total non-protein nitrogen.

In the soybean seed and other protein-storing systems, protein probably serves a dual function. Some is in enzymes and other metabolic components; the remainder, we postulate, is metabolically inactive storage protein. The storage protein provides an energy reserve and carbon skeletons in a role comparable to those of starch and fat, but is not metabolically active as are the proteins in leaves, roots, etc.

Large structures, identified histochemically as protein, appear in electron micrographs of developing cotyledons about 35 days before maturity and increase in number until they nearly fill the cells at maturity. These structures are considered to contain mostly storage protein, but the presence of some metabolic protein is indicated by small inclusions interpreted as lipoidal material. Other particulate matter identifiable in the micrographs also show the dual nature of protein in developing seeds.



Metabolic measurements on immature seeds reflect the changing status and distribution of nitrogen and protein during development. Oxygen uptake per seed parallels seed weight until the start of ripening. But when expressed on a weight or protein basis, it falls from an early rate of several thousand microliters of oxygen per gram dry weight to a few hundred at the start of ripening and to virtually zero at maturity. The decline during ripening is correlated with loss of moisture, but particulate protein decreases percentage-wise throughout development and on a per-seed basis during ripening. Oxidation by isolated mitochondria show trends similar to oxidation of whole seeds or cotyledons and account for a third to a half of intact cotyledon respiration.

The cytoplasmic protein in the centrifuge fractions referred to as "mitochondria" and "microsomes," i.e., those precipitating at about 15,000 g and 50,000 g, respectively, is interpreted as metabolic protein. Most of that in solution at 50,000 g is considered storage protein. The protein in mitochondria and microsome fractions generally parallels seed weight until ripening begins, then declines rapidly while soluble (storage) protein increases during the ripening process.

#### References

1. Bils, R. F. Ph.D. Thesis. University of Illinois. 1960.
2. Bils, R. F., and Howell, R. W., Crop Science. 1963 (in press)
3. Galitz, D. S., Ph.D. Thesis, University of Illinois. 1961.
4. Ohmura, T., and Howell, R. W. Physiologia Plantarum. 15:341-350. 1962.

## Discussion

- Dr. Sidney J. Circle: Would you comment on the fate of the starch (mature soybeans have none).
- Dr. Robert W. Howell: The starch serves as a reserve carbohydrate and makes up for the environmental changes occurring while the seed is developing.
- Mr. K. T. Holley: How do you define maturity in the soybean?
- Dr. Robert W. Howell: When the majority of the pods are brown, and the moisture content of the seeds is 10-12%.
- Dr. Aaron M. Altschul: How were the beans ground?
- Dr. Robert W. Howell: In sucrose, the usual procedure.
- Miss Mary L. Rollins: How did you determine which areas contained lipid?
- Dr. Robert W. Howell: The areas were empty when lipid solvents were used and, also, they were stained by Sudan.

## INTRACELLULAR LOCATION OF ZEIN IN MAIZE

Donald N. Duvick  
Pioneer Hi-Bred Corn Company  
Johnston, Iowa

The mature maize caryopsis is, like other angiosperm fruits, primarily an embryo with accompanying storage tissues which furnish sustenance to the germinating seed. As in nearly all grasses, the endosperm is the predominant storage tissue. In the embryo, a single cotyledon is modified into the structure known as the scutellum, an haustorial organ as well as a storage organ. The remainder of the embryo plus the scutellum are known collectively as the germ. The germ is derived from the union of a sperm nucleus with one of the three egg nuclei, and is therefore diploid. The endosperm consists of two clearly differentiated tissues: the single enveloping layer of aleurone cells, and the remainder. The endosperm arises from the union of the second sperm nucleus and two egg nuclei and is therefore triploid in each of its cells. The endosperm of the corn kernel can visually be divided into two distinctive tissues in most types of corn: the horny or corneous endosperm (also called "hard starch region") and the floury endosperm (also called "soft starch region"). In Flint corns the horny endosperm completely surrounds the kernel, except over the face of the germ, and is proportionately large. In Flour corns the horny endosperm is either absent or is present only as small shoulders near the base of the kernel. In Dent corns, typical of most of the corn of commerce, the horny endosperm is absent over the crown portion of the kernel, the size of the non-horny area varying with the variety of corn. This non-horny area is also depressed and wrinkled to a greater or lesser degree.

Cytologically the endosperm can be differentiated still further. At the base of the endosperm are elongated, crushed remnants of the cells of a rudimentary haustorial system which nourished the growing kernel. This system extends with some modification to the center of the endosperm and across the face of the scutellum. Cell sizes and shapes in the endosperm vary in a regular way, and various particulate components of the endosperm cells also vary in regular fashion from one region of the endosperm to another. These will be described in more detail in a later section.

### Chemical composition of the corn kernel

Chemically speaking, the general contents of the corn kernel are quite well known. The major portion of the kernel consists of carbohydrates, more specifically starch, and amounts to 70 - 80% of the moisture-free kernel. Approximately 10% of the kernel is composed of proteins and about 5% consists of oil. The remainder of the kernel (5-10%) consists of fibrous components, sugars, free amino acids and polypeptides (Curtis and Carle, 1946; Hopkins, et al., 1903). The relative proportions of the various components vary with variety and with conditions under which the seed was grown.

The two major divisions of the kernel: endosperm and germ, are visibly different in composition and likewise the relative proportions of carbohydrates, proteins and oils in the two structures are different. Germs average about 35% carbohydrates, 20% protein and 35% oil, whereas endosperms average about 90% carbohydrates, 8% proteins and less than 1% oil (Hopkins, et al., 1903; Hamilton, et al., 1951).



Further, it is known that the major types of proteins found in the germ are albumins and globulins (50% or more) with probably 30 - 40% of the remaining proteins being of the glutelin solubility type (soluble in weak alkali) and not more than 5% of the proteins of the zein solubility type (soluble in 70 - 80% alcohol). In contrast, about 45 - 50% of the endosperm proteins are zein and most of the remainder are probably glutelins, although some data indicate that as much as 25% of the endosperm proteins are of the globulin solubility type (Bressani and Mertz, 1958; Hamilton, et al., 1951). And finally, the protein of horny endosperm regions has a much higher proportion of zein than does the protein of the floury endosperm regions (Hamilton, et al., 1951). Thus it is clear that the maize caryopsis is chemically differentiated into several regions.

The general distribution of protein types between germ and endosperm is about what might be expected. The germ should be expected to be more active enzymatically than the endosperm, and it is therefore not surprising to see that the germ has a large proportion of its protein in the form of salt and water-soluble proteins. Such proteins typically are enzymatic in function. The alkali- and alcohol-soluble proteins which make up the bulk of the endosperm protein are no doubt metabolically inert until various hydrolytic enzymes secreted by the aleurone and the scutellum of the germinating seed break them down into metabolically useful amino acids, polypeptides or other nitrogen residues.

Looking more closely at the zein and glutelin proteins of the endosperm, we find that as we attempt to locate them in certain endosperm

cells, or in certain structure of the endosperm cells, the degree of precision lessens. In the first place, we should note that there is some doubt as to whether the zein and glutelin "families" exist in the cell as separate entities. It is possible that the arbitrary methods for extracting zein and glutelin merely sort into two groups something that the cell has synthesized as one functional entity, or even as one species of molecule.

Amino acid analyses have shown that zein and glutelin fractions differ in relative amount of several amino acids (Hansen, et al., 1946; Osborne and Clapp, 1907). Zein is notorious for having neither tryptophan nor lysine, for example, while glutelin has about 3% lysine and at least some tryptophan.

Electrophoresis studies of zein and glutelin extracts have shown that both of these protein extractions were inhomogeneous (Mertz, et al., 1958). Zein contained at least five components. Glutelin had two components and both of these corresponded in mobility to components found in zein. However, zein was composed predominantly of one component, and glutelin likewise was predominantly one component. The major zein component was different from the major glutelin component.

One further type of evidence indicates that glutelin and zein are probably distinct entities in the endosperm. Analyses made at successive stages of kernel development show that most of the glutelin protein is synthesized in the first two or three weeks after pollination whereas the synthesis of zein in the kernel continues throughout the entire period of kernel development, with the major deposition occurring in the latter portion (Watson, 1949; Zeleny, 1935; Bressani and Conde, 1961).

## Cytological studies of endosperm cell contents

One approach to learning about the introcellular location of the endosperm proteins is to follow, developmentally, the origin and growth of cell contents in the various locations of the endosperm. Histochemical tests can give some indication of the general chemical composition of the various cell contents.

A brief morphological and cytological summary of the developing endosperm (Randolph, 1922; Duvick, 1951, 1955, 1961) will provide a background for the descriptions of intracellular inclusions.

The endosperm is coenocytic during the first three or four days after pollination; that is, no cell walls are laid down between the dividing nuclei. At about three days, the endosperm becomes cellular; divisions continue apparently at random throughout the entire endosperm until about six days, at which time cells in the base of the endosperm, in contact with maternal vascular tissues, stop dividing and differentiate into a primitive conducting tissue. In another day or two, cells in the center of the endosperm also cease their mitotic activity and enlarge. By this time (seven to nine days after pollination) mitotic activity is limited to four or five layers of peripheral cells. These cells are rectangular in shape and divisions take place either at right angles to the surface of the endosperm, or parallel with it.

At about 10 to 15 days after pollination, mitotic activity reaches its peak; then, quite rapidly, (1) the outer layer of cells is visibly differentiated as the aleurone layer, and (2) the number of dividing cells



drops quite rapidly, although some divisions continue for a time in those layers just under the aleurone. A progression of cessation of mitoses moves downward and outward in a wave-like fashion: division ceases first in the cells in the silk-scar region of the kernel (near the apex on the germinal side of the endosperm) and sweeps down to the basal regions. The extreme base and the center of the endosperm have previously ceased cell division, as stated above. All divisions have ceased by about 21 days after pollination.

At about the same time that mitotic activity reaches its peak, the first visible starch is seen in endosperm cells. The first starch (at 9 to 13 days after pollination) is seen in cells in the crown region of the kernel (under the silk scar). Initiation of starch synthesis in endosperm cells also spreads with a wave-like progression. Starting at a point several cell layers in from the periphery of the endosperm, the wave sweeps towards the periphery and at about the same time, toward the base of the endosperm (Fig. 1). Aleurone cells, on the extreme periphery, do not form starch (except rarely) and neither do the basal absorbing cells, nor the innermost, extremely large and vacuolate cells of the endosperm.

The starch grains are first formed as an enlarged knob on some of the numerous filamentous inclusions visible in all endosperm cells. The cells destined to form starch show enlarging of one of the knobs on many of their filaments a few days before the starch can be detected with iodine stain, and these filaments with enlarged knobs are typically clustered over the surface of the nucleus a day or two before they begin to fill with starch (Duvick, 1955).



Endosperm cells, just prior to visible starch synthesis, also have in them many granular bodies, which exhibit rapid cyclosis. These bodies are about 1 micron or less in diameter. The enlarged filament knobs (by way of contrast) are about 1 - 2 microns in diameter when they first deposit starch.

Cell enlargement proceeds throughout the endosperm in a manner similar to the other progressions described. Enlargement begins in the center of the endosperm, spreads to the outer cells first in the upper portion of the endosperm, and then sweeps down to the base of the endosperm, always with a simultaneous progression from center to periphery.

Measurement of diameters of starch grains in equivalent cells of the endosperm (after mitoses have ceased) in samples gathered at successive stages of development allows one to plot the rate of growth of starch grains in selected areas of the endosperm. Counts of starch grains per cell in a cross section of the cell can give estimates of the changes in numbers of starch grains per cell in selected regions of the endosperm.

Such counts have shown that a given cell will initiate at one time (within a day or two) all the starch grains it contains at kernel maturity. Therefore the curve for average increase in size of the starch grains will describe the timing and the rate of increase of starch content of a given endosperm region. Compilations of a number of such curves for various types of corn have demonstrated that the accumulation of starch in the maize endosperm can be described as a series of progressions or waves much like those for the above described phenomena of cessation of cell division, initiation of starch synthesis, and cell enlargement.

Within any one endosperm region, the inner endosperm cells are first to reach a maximum rate of starch synthesis, and a wave of maximum rates moves from inner to outer cells. The magnitudes of these maximum rates become successively less as the wave expands to the periphery of the endosperm (Fig. 2). The length of the period of synthesis (on a per cell basis) becomes successively longer as the wave goes from center to periphery, until about the 13th cell from the periphery. From there on it stays about the same. At about the 10th to the 13th cell layer, counting in from the aleurone, the optimum combination of rate and length of period coincides to produce the largest starch grains. Proceeding either to the center or to the periphery of the mature endosperm from cells 10 - 13 one finds that starch grains are progressively smaller.

As in other progressions described, a wave of maximum rates of synthesis moves toward the base of the endosperm at the same time as the maxima are moving toward the periphery. Thus, cell number 13 (counting in from the aleurone) in the crown region of the endosperm reaches its maximum rate of starch synthesis several days before cell number 13 in a more basal region of the endosperm (Fig. 3). In general the length of the synthetic period is progressively longer, going from upper to lower regions of the endosperm.

In the mature endosperm, starch grains usually are largest in the shoulders and rear of the kernel (the abgerminal portion, and on both sides of it) and are smallest in the crown region. In most dent corns, cells in the portion of the crown which dents form little or no starch.

Although those cells commence initiation in their proper turn, starch synthesis ceases abruptly at such an early date that the final starch grain size is very small.

Within a week or two after starch synthesis has begun in a given endosperm cell, an enlargement of some (but not all) of the small spherical granules in the cell begins. One cannot be sure that the granules which enlarge are the same as the rapidly moving granules observed in equivalent cells a week or two earlier, but it is quite certain that the enlarging granules do not develop from the filamentous inclusions of the type that gave rise to starch grains.

These enlarging granules give positive cytochemical tests for protein, and negative tests for starch and fats (Duvick, 1961) and will henceforth be referred to as "protein granules".

The pattern of enlargement of the protein granules in various endosperm regions exhibits the simultaneous outward and downward progression characteristic of starch grain enlargement. Like starch grain enlargement, the zone of protein granule enlargement originates in the crown area, more or less beneath the silk scar, and spreads downward and outward. The length of the period of enlargement grows longer, going from inner to outer cells, and from upper to lower regions of the endosperm. However, unlike starch grain enlargement, the rates of enlargement of the protein granules grow progressively greater as one goes from inner to outer cells of the endosperm (Fig. 4). (Starch grain rates were progressively smaller.) This means that the average diameters of the protein granules in each cell become progressively greater, going from inner to outer cells of the endosperm. Accurate counts of numbers of protein granules per cell are difficult to



make, but the numbers appear to be greater in the outer cells. However, since the outer cells are smaller and the size of the granules is greater, the increase in numbers of granules may be more apparent than real. It is obvious, however, that the proportion of cell volume occupied by protein granules becomes progressively greater, going from inner cells to outer, and that the relative volume occupied by starch grains becomes progressively less.

As stated above, protein granule enlargement in the endosperm begins at about 15 - 20 days after pollination. Considering the endosperm as a whole, it is hard to say when the maximum amount of protein granule enlargement is occurring, for there is a constant succession of initiations, peak periods, and cessations of enlargement moving downward and outward throughout the endosperm. However in Dent corn the cells in which the granules become the largest and the cells making up the greatest bulk of the endosperm are at the highest rate of protein granule enlargement during the last two-thirds of the endosperm maturation period (Fig. 5). In contrast, the bulk of starch grain enlargement in Dent corn takes place in the middle one-third of the endosperm maturation period.

When cells of the mature endosperm are examined cytologically and cytochemically, they show that a typical storage cell contains: (1) starch grains, (2) protein granules, (3) an optically clear proteinaceous matrix, (4) a crushed, bedraggled nucleus, and (5) a few filamentous and granular inclusions visually no different from those seen in mitotic cells of the very young endosperm.



Considering the first three classes of inclusions, one can best describe the cell (after hematoxylin staining) as appearing like a box filled with white marbles (the starch grains) with gray buckshot dispersed in the spaces between the marbles (the protein granules) and the whole collection imbedded in a clear glue which has been poured over the entire mass and then hardened. In the outermost endosperm cells the hypothetical buckshot can be pictured as being nearly as large as the marbles. In most horny endosperm regions the marbles can be pictured as having been rather soft and subjected to compression so that they are more or less flat-sided. In the floury endosperm cells the "buckshot" would be quite small relative to the "marbles"; the "marbles" would be spherical and all contents would be rather loosely arranged. Further, the glue in the floury endosperm cells may be pictured as having shrunk as it hardened. It was then insufficient to fill all the spaces between starch grains and protein granules, and so it ruptured in many places at a certain stage in the drying-shrinking process. The fractured surfaces are rough and highly refractive. They scatter light and give a "frosted glass" look to the face of the split cell. This is the reason for the chalky appearance of the floury endosperm areas in a split kernel. The "glue" (clear protein) is intact in the horny endosperm cells and the tissue is optically more or less homogeneous and is able to transmit light quite well.

The description given of the development of inclusions in the maize endosperm has shown the appearance and increase in bulk of two classes of inclusions: (1) starch grains and (2) protein granules. A third substance: (3) the "clear protein" matrix is also described as present in the mature endosperm. The starch grains as a whole enlarge most during the

central third of the endosperm maturation period; the protein granules during the latter two-thirds of the period. The time of appearance and rate of increase in quantity of the clear protein is not known.

#### Evidence for specific intracellular locations of zein and glutelin

Since the picture presented here shows two intracellular locations for endosperm storage protein: (1) the granules and (2) the "clear protein" matrix and since chemical data indicate there are two classes of endosperm storage protein: zein and glutelin, it is tempting to try to associate one of the chemical entities with the protein granules and the other with the "clear protein". The evidence so far presented has shown that the granules' grand period of growth roughly coincides with the period of greatest zein accumulation, and that it definitely does not coincide with the period of glutelin accumulation. The period of glutelin accumulation, incidentally, roughly coincides with the period of mitotic activity in the endosperm.

Several histochemical tests specific for certain amino acids were performed on sectioned kernels (Duvick, 1951 and 1961). A test for arginine was negative in protein granules in all regions but positive, although weak, in the clear protein of all regions. Zein contains about 1% arginine in comparison to about 7% arginine in glutelin (Osborne and Clapp, 1907). Aleurone cells, incidentally, gave a strong positive test for arginine. Two types of test for tryptophan gave a faint positive reaction distributed fairly evenly over the whole endosperm. The reaction was so weak that it could not be localized in any particular inclusions of the cells. However, the even distribution of color corresponded with the distribution of clear

protein throughout the endosperm and did not correspond with the increase in relative bulk of protein granules, going from center to periphery of the endosperm. Glutelin has about 1% tryptophan, whereas zein has essentially none (Lloyd and Mertz, 1958).

When freezing microtome sections of mature endosperms were immersed in 0.2% sodium hydroxide for as little as 30 minutes the clear protein was largely dissolved, allowing the starch grains and protein granules to fall from the cells. Neither the clear protein nor the protein granules were affected when endosperm sections were immersed in 70% alcohol, nor when immersed in a 5% sodium chloride solution (Duvick, 1961). Glutelin by definition is soluble in dilute alkali but not in alcohol. Zein by definition is soluble in alcohol. Although zein extractions may be made with alcohol as the first solvent, the grain is ground. If in microtome sections the clear protein surrounding each protein granule is insoluble in alcohol the encased granule will be protected from the solvent action of the alcohol. On the other hand the clear protein will be fully exposed to the action of the alkali solution.

When kernels with a high percentage of protein are compared with kernels of a low protein percentage, it can be seen that the protein granules of the high protein corn are much larger than those of the low protein corn (Duvick, 1961). Numerous chemical investigations, some dealing with the corn examined cytologically, have shown that an increase in the zein fraction of high protein corn is the principle cause of the increase in protein content of the corn, irrespective of whether the increased protein content is a result of breeding or of soil fertilization (Hamilton, et al., 1951; Watson, 1949; Hansen, et al., 1946).



Finally, it has been shown that the proportion of zein in horny endosperm is much greater than that in floury endosperm (Hamilton, et al., 1951); this corresponds with the increase in size of protein granules going from center to periphery of the endosperm. It should be re-emphasized, however, that the difference between floury and horny endosperm is a quantitative one, both chemically and cytologically. It only appears to be qualitative to the unaided eye because of the threshold point at which the clear protein does or does not rupture when the kernel dries down, at maturity.

### Conclusions

Thus, for the following reasons, it appears that the protein granules in endosperm cells may be the major storage organelles for zein, and the clear protein matrix of the endosperm cells may be the major storage location for glutelin:

- (1) Time of zein accumulation in the kernel agrees with time of most of protein granule growth, but time of glutelin accumulation does not.
- (2) Histochemical tests for some of the amino acids present in zein and glutelin in widely different percentages have shown differences between protein granules and clear cytoplasm. The differences are explainable on the basis that the granules are mostly zein and the clear protein is mostly glutelin.
- (3) Weak alkali, a glutelin solvent, dissolves the clear protein, but alcohol, a zein solvent, does not.



(4) Protein granules of corn with high zein content are larger than those of corn with low zein content.

(5) Protein granules are larger and amount to a larger proportion of the cell in the endosperm regions with high zein content (horny endosperm) than in endosperm regions with low zein content (floury endosperm). Conversely, they are smaller and proportionately less in regions with high glutelin content than they are in regions with a low glutelin content.

#### Immunological analysis of corn kernel proteins

The currently popular immunological technique has been used in our laboratory during the past couple of years for studying the salt and water soluble proteins of the corn kernel. In exploratory tests, I have found that proteins extracted with a buffered 1% salt solution from ground entire kernels are immunologically distinct from those extracted with identical methods from pollen, leaves, sprouts and roots of corn plants. The proteins of each of these other organs are immunologically distinct, also. Studies of developing kernels have shown that there appear to be changes (in time) in proportion but probably not in kinds of several proteins. The major phase of the research is aimed at discovering differences between genotypes, in any tissue that will serve. Comparisons of protein extracted from mature kernels have shown that there are definite quantitative differences between inbred lines, with regard to at least two salt soluble proteins. It is not known where these are, in the kernel, nor what their nature or functions might be.

## References

1. Bressani, R. and Conde R., Changes in the chemical composition and in the distribution of nitrogen of maize at different stages of development. *Cereal Chem.* 38:76-84. 1961.
2. Bressani, R. and Mertz, E. T., Studies on corn proteins IV. Protein and amino acid content of different corn varieties. *Cereal Chem.* 35:227-235. 1958.
3. Curtis, J. J. and Earle, F. R., Analyses of double-cross hybrid corn varieties produced on farms. *Cereal Chem.* 23:88-96. 1946.
4. Duvick, D. N., Development and variation of the maize endosperm. Ph.D. Thesis, Washington U., St. Louis, Mo. 1951.
5. Duvick, D. N., Cytoplasmic inclusions of the developing and mature maize endosperm. *Amer. Jour. Bot.* 42:717-725. 1955.
6. Duvick, D. N., Protein granules of maize endosperm cells. *Cereal Chem.* 38:374-385. 1961.
7. Hamilton, T. S., Hamilton, B. C., Johnson, B. C. and Mitchell, H. H., The dependence of the physical and chemical composition of the corn kernel on soil fertility and cropping system. *Cereal Chem.* 28:163-176. 1951.
8. Hansen, D. W., Brimhall, B. and Sprague, G. F., Relationship of zein to the total protein in corn. *Cereal Chem.* 23:329-335. 1946.
9. Hopkins, C. G., Smith, L. H. and East, E. M., The structure of the corn kernel and the composition of its different parts. *Ill. Sta. Bull.* 87:77-122. 1903.
10. Lloyd, N. E. and Mertz, E. T., Studies on corn proteins. III. The glutelins of corn. *Cereal Chem.* 35:156-168. 1958.
11. Mertz, E. T., Lloyd, N. E. and Bressani, R., Studies on corn proteins. II. Electrophoretic analysis of germ and endosperm extracts. *Cereal Chem.* 35:146-155. 1958.
12. Osborne, T. B. and Clapp, S. R., Hydrolysis of proteins in maize. *Amer. Jour. Physiol.* 20:477-493. 1907.
13. Randolph, L. F., Developmental morphology of the caryopsis in maize. *Jour. Agr. Res.* 53:881-916. 1936.
14. Watson, S. A., An agronomic and biochemical comparison of four strains of corn which differ widely in total grain nitrogen. Ph.D. Thesis, U. of Ill. Urbana, Ill. 1949.
15. Zeleny, L., The distribution of nitrogen in the seed of *Zea mays* at different stages of maturity. *Cereal Chem.* 12:536-542. 1935.

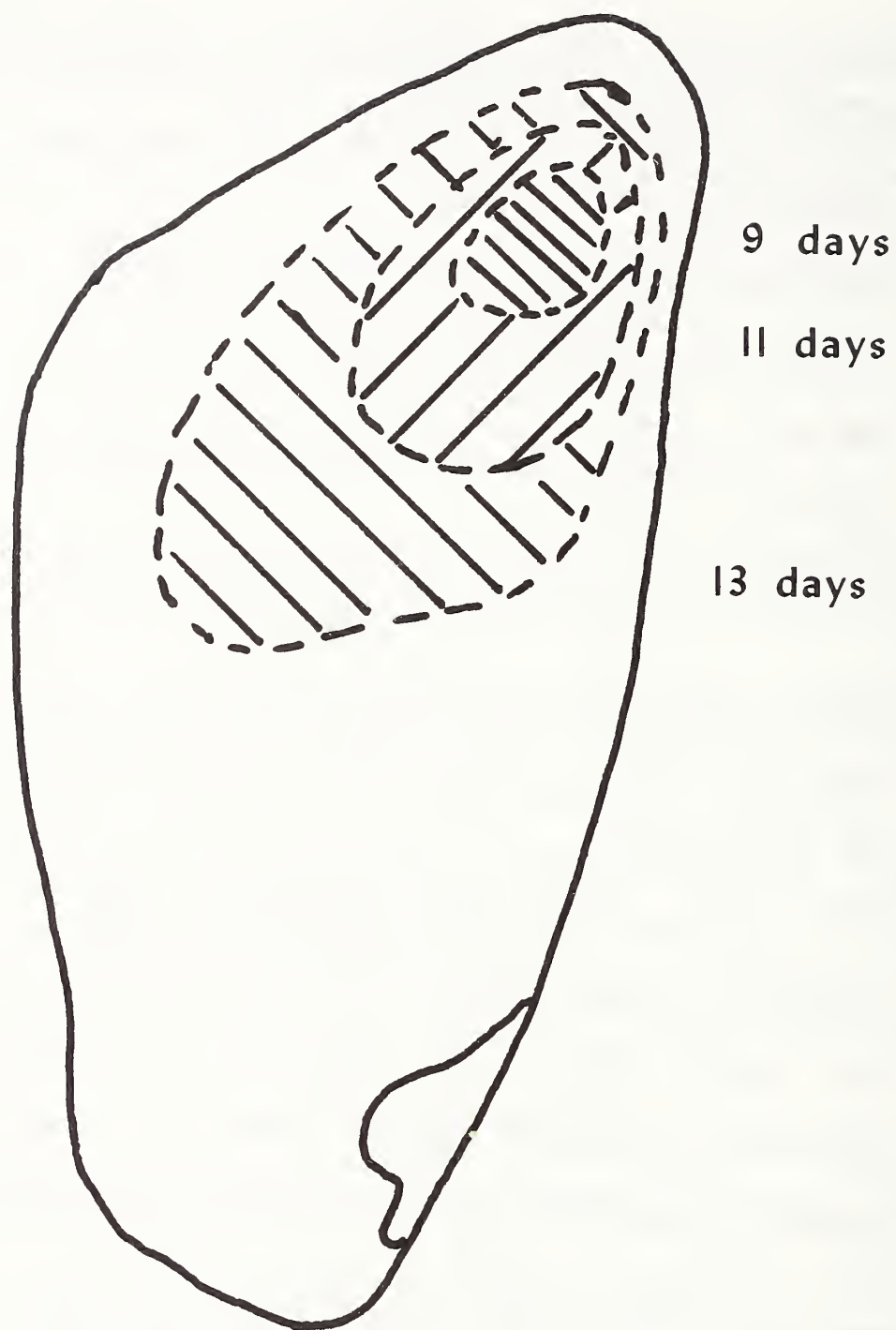


FIGURE 1

Cross-section of immature kernel (schematic) illustrating successive stages of enlargement of the region of starch-containing cells.

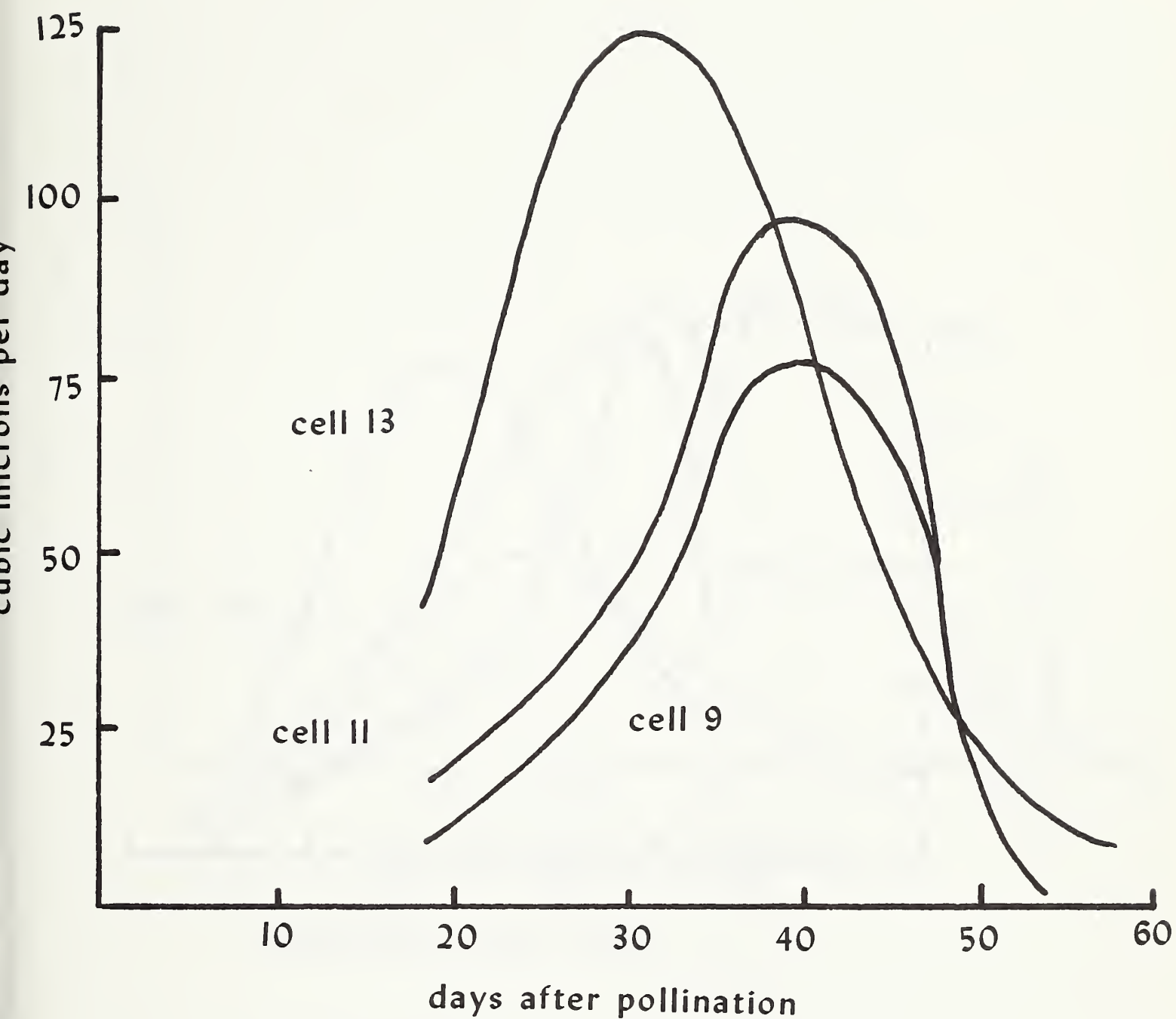


FIGURE 2

Rates of increase in volume of starch grains in three different cell layers of endosperm (central abgerminal region) of Gourdsed Southern Dent corn (from Duvick, 1951).



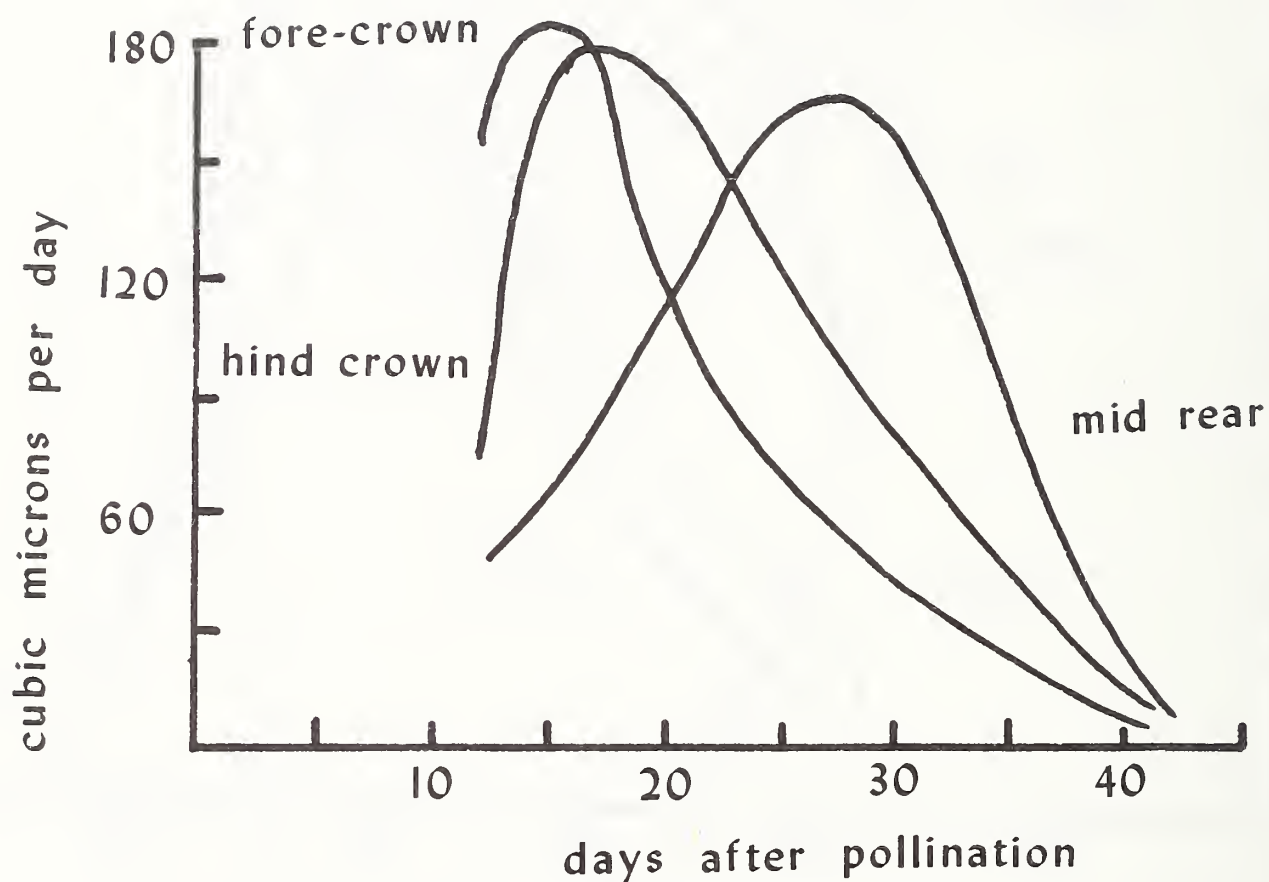


FIGURE 3

Rates of increase in volume of starch grains in cell layer no. 13 of three endosperm regions of Longfellow Flint (from Duvick, 1951).

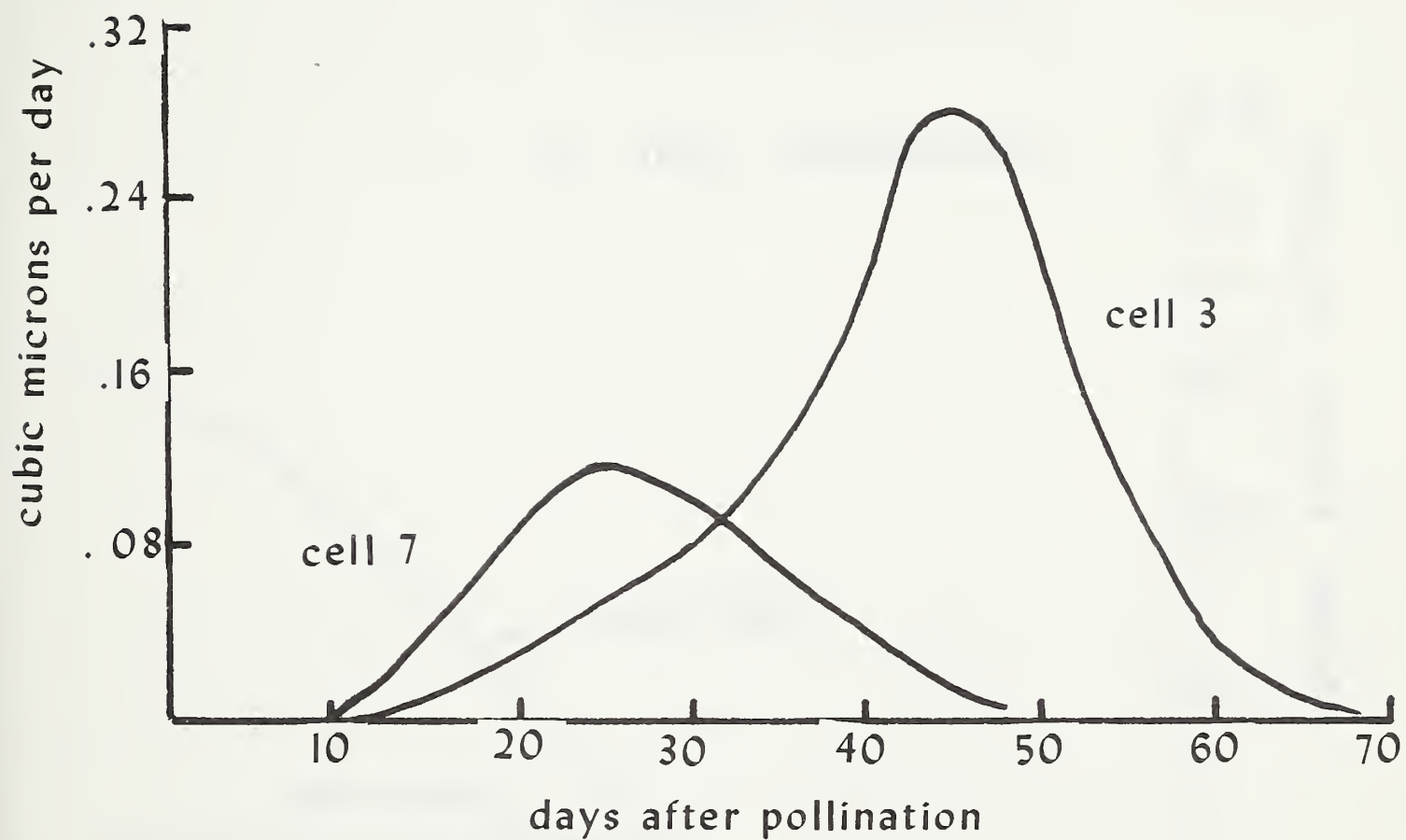


FIGURE 4

Rates of increase in volume of protein granules in two different cell layers of endosperm (central abgerminal region) of Longfellow Flint corn (from Duvick, 1951).

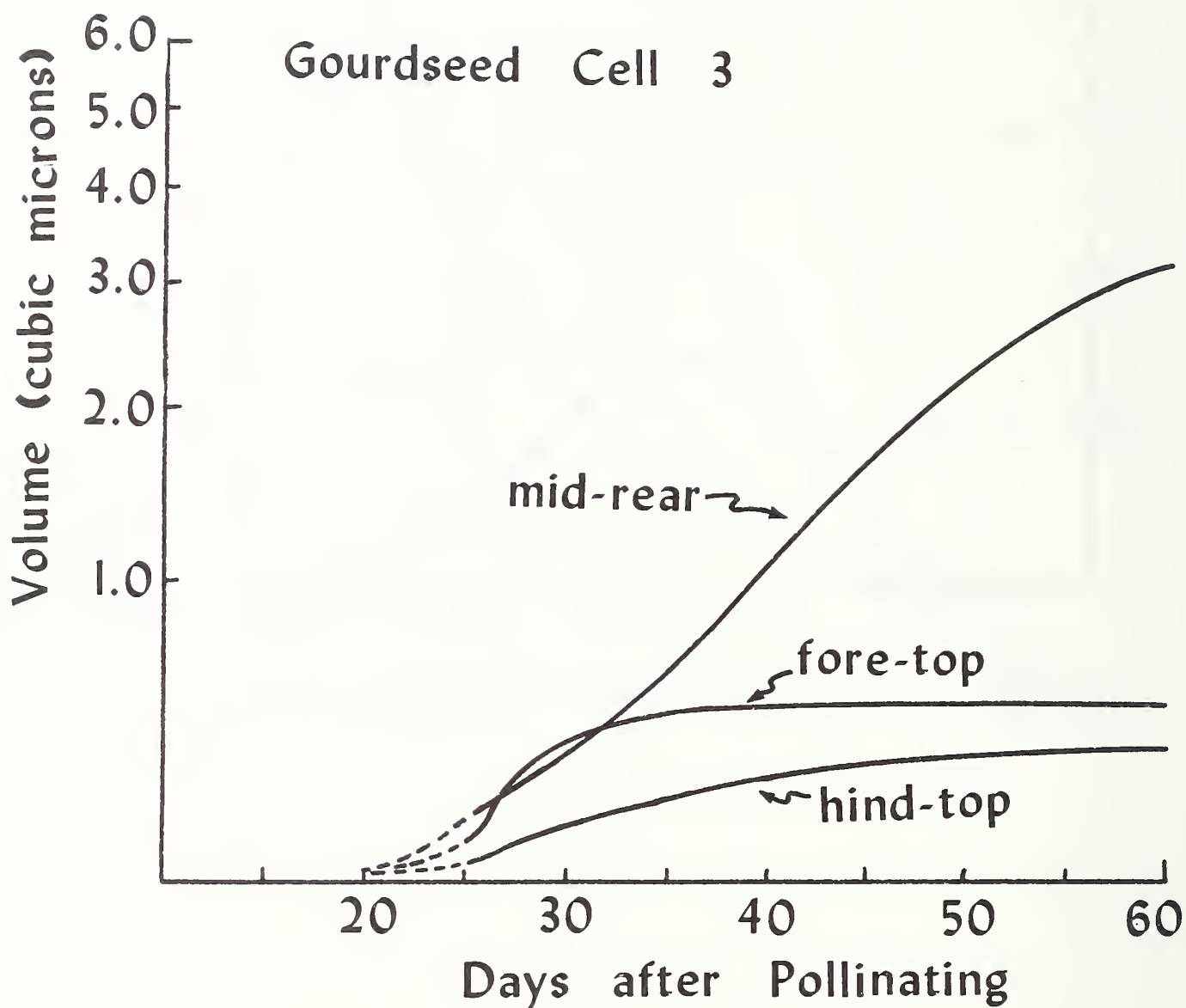


FIGURE 5

Increase in volume of protein granules in three endosperm regions of Gourdseed Southern Dent corn, in cell layer no. 3 (from Duvick, 1961).

## Discussion

Miss Mary L. Rollins:

How are you going to use your information?

Dr. Donald N. Duvick:

We're trying to learn more about what can be expected when breeding corn for different levels of protein content. There may be physiological and cytological limits to what can be accomplished.



# FRACTIONATION OF PARTICULATE PROTEINS OF THE PEANUT COTYLEDON

by

W. J. Evans, A. A. Woodham, W. B. Carney,  
J. M. Dechary, and A. M. Altschul

It has been generally considered that most of the protein in parenchyma cells of high protein content exists in particles visible by light microscopy; these have been described as ergastic inclusions and have been called aleurone grains. The evidence that these are protein particles is based on their response to protein stains. At this meeting Duvick reported protein bodies in the maize endosperm, Varner reported protein bodies in pea seeds, and Cherry described the changes in protein bodies of peanuts during germination as an ordered series of events leading to degradation of the protein in the cotyledonary tissue.

Perhaps stronger evidence that these particles, visible by microscopy, are indeed high in protein content comes from subcellular fractionation of the contents of parenchyma cells. We shall discuss two approaches: nonaqueous fractionation as done by Dieckert and Snowden in this laboratory, and sucrose fractionation by techniques adapted to seed tissue by Woodham and by Evans and Carney. The various fractions were analyzed by DEAE cellulose chromatography described in an earlier paper by Dr. Dechary; as you will remember, he differentiated the soluble proteins into four groups based on the salt concentration in the eluate. Groups III and IV, those most tightly bonded to the cellulose, comprise the major portion of the proteins.

Nonaqueous fractionation. Dieckert et al. fractionated components of the peanut cotyledon in nonaqueous solvents. The fractions which were analyzed were a gift from Dr. Dieckert and Mr. Snowden. Peanut cotyledons disintegrated in vegetable oil and the density of the medium was adjusted by adding carbon tetrachloride. Separations according to density yielded the following fractions: two protein-rich, cell wall, starch grain, a light material which appears as a fine network when observed under the electron microscope, and a parenchyma tissue.

The effluent diagrams of the total soluble proteins of the peanut cotyledon and of the proteins of each of the two protein-rich fractions are shown in Figure 1. No significant difference is observable between all three patterns; they all show the same general pattern of four groups. There are minor differences in the structure of the peaks and these may be significant. In view of the complexity of the mixture and the known high tendency for interaction among seed proteins, it might be expected that the major protein fractions would dominate the chromatographic pattern. It is clear that the major proteins (groups III and IV) are present in all three patterns.

With these reservations, it can be concluded that the bulk of the proteins of the cotyledon can be accounted for as the proteins in the two protein-rich fractions. It is interesting to note that even though the two protein-rich fractions differ from each other in density and in content of nitrogen and phytic acid, this is not reflected in any profound difference in their protein patterns. It would seem, therefore, that

little if any fractionation of the major proteins has been achieved by collecting these particles. This is not surprising since this is nonaqueous fractionation technique; no opportunity is offered, therefore, for washing the particles and removing from them soluble proteins that might otherwise be fractionated away from the particles.

But the major point, namely that subcellular fractions can be isolated which are high in protein content and contain all the major proteins of the peanut cotyledon has been established. This therefore supports the indirect evidence from staining that there exist concentrations of protein in subcellular locations.

Altschul et al. demonstrated that in the presence of Carbowax 20M, 75% of the total proteins of the peanut were not solubilized in a buffer medium in which they are ordinarily dissolved. This was construed as additional evidence that the major proteins of the peanut exist in subcellular particles whose rupture was prevented by appropriate media. There was also the suggestion from their data that some fractions of particulate proteins could be separated in 0.25 M sucrose media. Varner and Schedlowsky were able to fractionate the total proteins of the pea seed by preventing dissolution of some of the subcellular particles.

Fractionation in Sucrose Solution. This first procedure was developed in cooperation with Dr. A. Woodham of the Rowett Institute. Peanut cotyledons were homogenized in four times their weight of 0.25 M sucrose in 0.05 phosphate buffer, pH 7.0; debris and starch grains were removed by spinning at 1000 x g for 5 minutes. The supernatant was refrigerated overnight and then recentrifuged at 1000 x g for 5 minutes. The pellet



was resuspended and recovered by centrifugation twice more, then it was layered over 50% sucrose in phosphate buffer and centrifuged at 10,000 x g for 10 minutes. The protein at the interface between the two sucrose solutions was recovered and will be denoted as sucrose fraction (a). A portion was suspended in 5% NaCl and dialyzed against phosphate buffer in preparation for DEAE cellulose chromatography, another portion was dialyzed against water and freeze dried. The dialyzed material had a nitrogen content of 3.1%.

The low value for protein in this particulate fraction suggested that perhaps some of the protein was being solubilized in the buffer. A second fractionation was undertaken with Dr. Evans and Mr. Carney without controlling the pH of the sucrose solution and in the absence of added salts. Peanut cotyledons were presoaked at 3° C. in 0.25 M sucrose and then macerated in an Omnimix. Starch grains and cellular debris were removed by centrifuging at 1000 x g for 10 minutes.

The second fraction brought down at 10,000 x g for 10 minutes was the one examined further. It was repurified by layering over 50% sucrose and recentrifuging at 10,000 x g for 30 minutes. One portion of the pellet was dialyzed in the appropriate buffer prior to chromatographic analysis, and another was dialyzed against water for nitrogen analysis. It contained 10.7% nitrogen; it is denoted as sucrose fraction (b).

The supernatant after removal of sucrose fraction (b) was centrifuged at 40,000 x g for 90 minutes, yielding a pellet and a clear supernatant.



That particulate protein may be fractionated in sucrose media is shown by comparing the elution diagrams with those for the total proteins shown in the one for the protein-rich fractions of Dieckert. In Figure 2 is the elution pattern of sucrose fraction (a). The elution pattern of sucrose fraction (b) is quite similar to the one shown in Figure 2, particularly in the complete absence of the peak which represents alpha-conarachin (elution at 0.17 M salt concentration). What is primarily observed is the major protein fraction of the soluble peanut proteins, the so-called group IV.

Further attempts at fractionation of the particles by centrifugation through various density gradients failed to change the pattern. This may mean that we have a fundamental particulate fraction or it is possible that some extension of particulate separation might simplify further the patterns in these slides.

In Figure 3 (solid line) is shown a chromatogram of a fraction of the total peanut protein which comes down at 40% saturation with ammonium sulfate; this has already been shown by Dr. Dechary; this has been classically called the arachin fraction. The correspondence between the elution pattern of this fraction with that isolated by subcellular fractionation in sucrose media is striking. Under the conditions of fractionation in ammonium sulfate, the proteins are retained as a group similar to that which is found in the particulate. It would seem that even after they are dissolved these proteins behave as if they were one large aggregate of protein separable only by such means as cellulose chromatography.

This phenomena is understandable in terms of the high degree of interaction between the various protein globulins of the peanut.

The supernatant after centrifugation of the sucrose homogenate at 40,000 x g for 90 minutes contains none of the major protein fractions (groups III and IV). This implies that there are several types of particulates containing protein; group III, which contains  $\alpha$ -conarachin, is in a different particle from the one isolated at 10,000 x g. (sucrose fraction (b)).

#### Summary

One of the difficulties in dealing with proteins of an entire tissue is the possibility that what is observed is an artifact of mixing the proteins during maceration of the tissue. Hence, there always is the question that the protein groups observed in the tissue extracts do not exist as such in situ. This problem is particularly acute when dealing with proteins of no obvious enzymic activity, as is true for the major seed globulins. One approach is to simplify the source material for protein extraction, and this was accomplished by isolating protein from a particle. The fact that similar elution patterns are obtained from ammonium sulfate fractions and from particle fractions would suggest that the study of the seed proteins in the peanut is not confounded by interaction between the major groups such as groups III and IV, even though there is reason to suspect interaction of proteins within these groups.

It would seem that the biochemical evidence supports the general assumption that the major proteins in peanut cotyledons exist in particulates. By nonaqueous techniques, particles have been obtained which have the same

size and similar morphological characteristics to the classical aleurone grains. These particles are rich in proteins, and moreover contain all of the major proteins of the peanut in a proportion similar, if not identical, to that of the entire cotyledonary tissue.

The fact that proteins may be fractionated by particle separation techniques would suggest either that these protein-rich fractions are compound particles consisting of several types of protein bodies separated from each other by maceration in sucrose medium, or that protein particles exist also in the interbody space in the cytoplasm.

The study of the origin of the major seed proteins might be considered simplified by identifying this problem with the synthesis and accumulation of particulates which can be **observed** microscopically, as well as by biochemical techniques and which may be isolated intact from macerated cells. A comparison of the proteins of these particulates with those of known particulates of metabolically-active tissue might throw light on the origin of these major seed proteins.

#### References

1. Dieckert, Julius W., Snowden, James E., Jr., Moore, Anna T., Heinzelman, Dorothy C., and Altschul, Aaron M. Composition of Some Subcellular Fractions from Seeds of Arachis hypogaea. J. Food Sci. 27: 321-325 (1962).
2. Dechary, J. M., Talluto, K. F., Evans, W. J., Carney, W. B., and Altschul, A. M. Alpha-Conarachin. Nature 190: 1125 (June 17, 1961).
3. Altschul, A. M., Snowden, J. E., Jr., Manchon, D. D., Jr., and Dechary, J. M. Intracellular Distribution of Seed Proteins. Arch. Biochem. Biophys. 95 (2): 402-404 (1961).



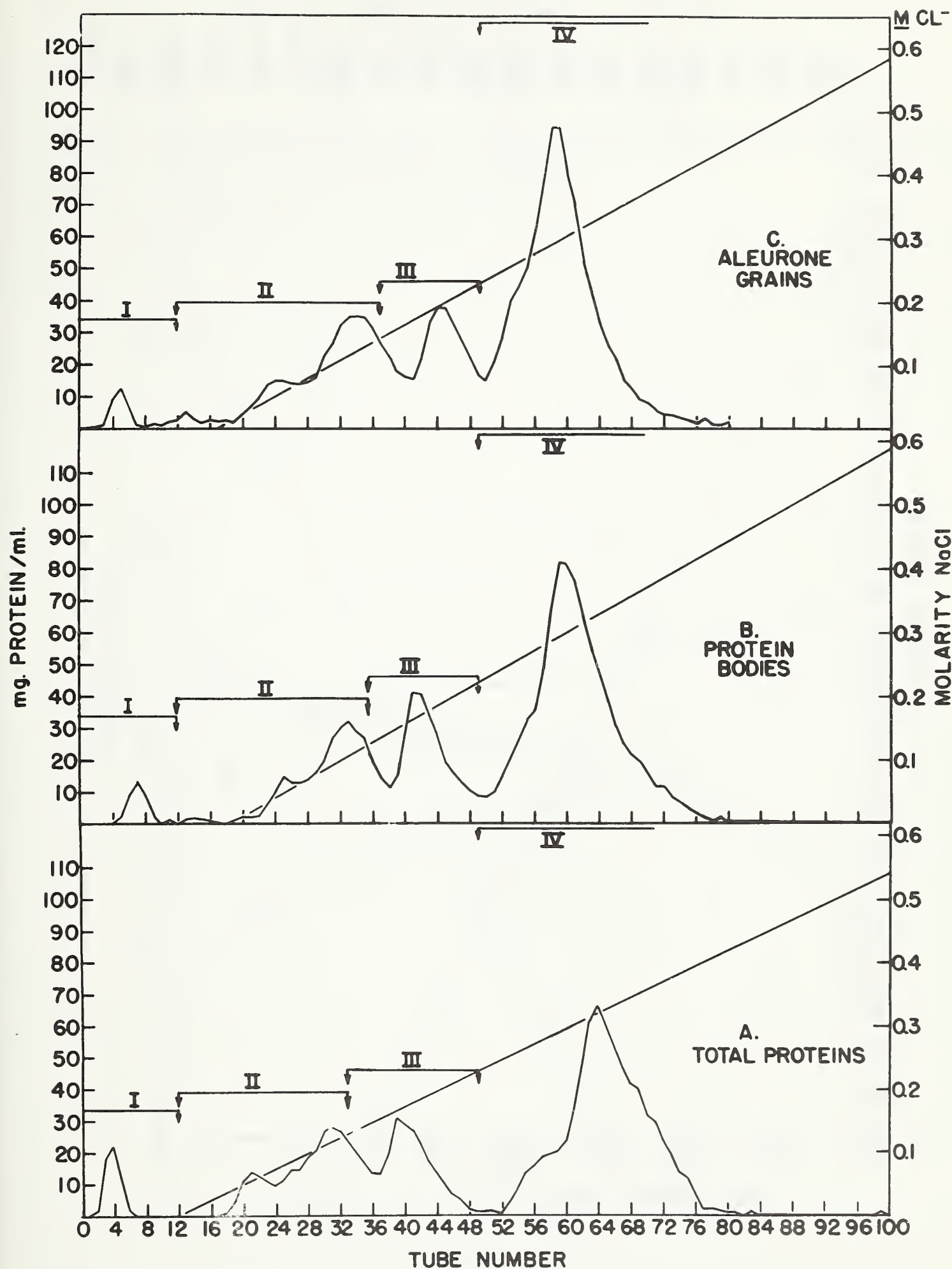


FIGURE 1  
Effluent diagrams of total soluble proteins of  
the peanut and of two protein-rich fractions.



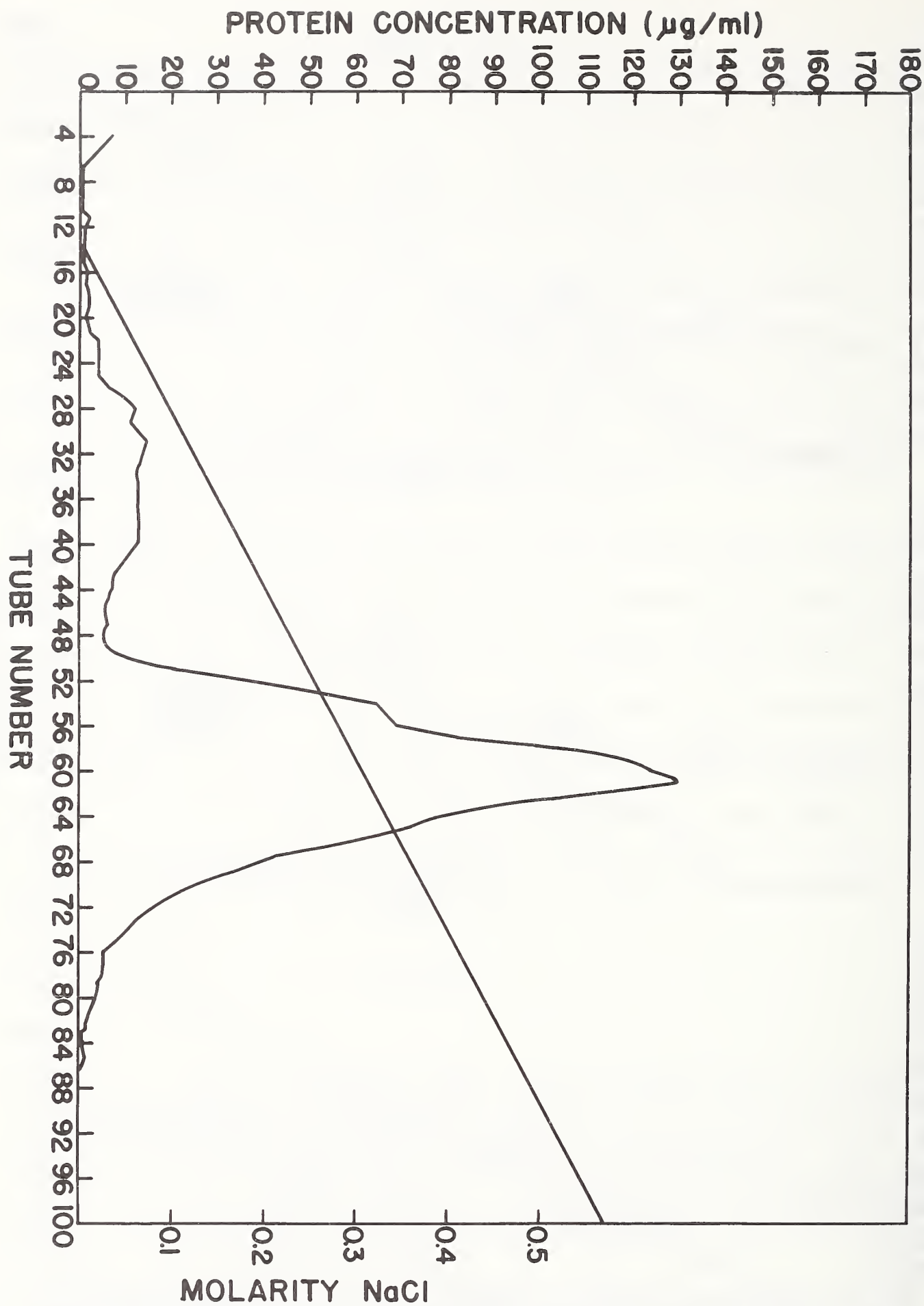


FIGURE 2  
Effluent diagram of proteins in sucrose fraction (a).

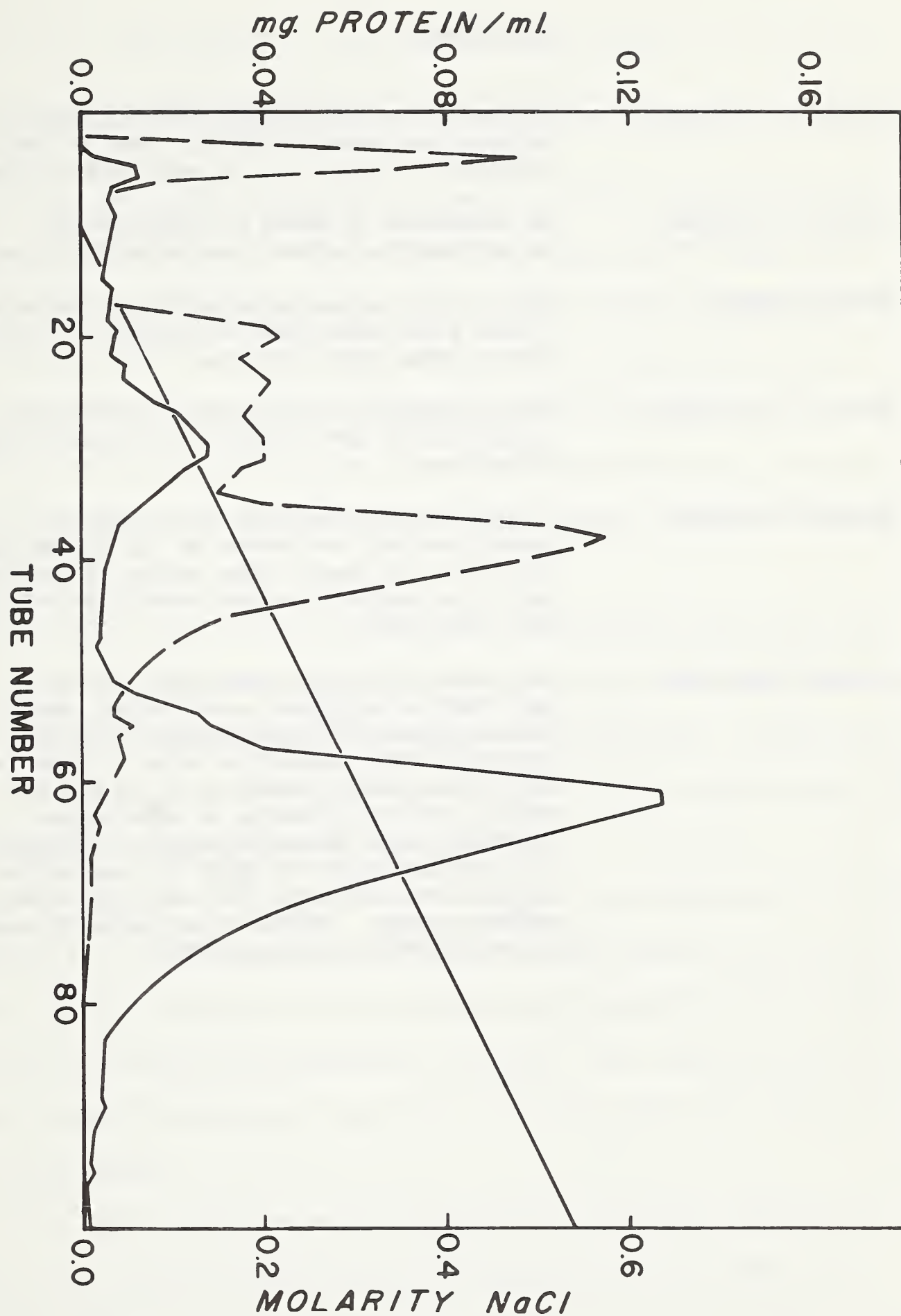


FIGURE 3  
Elution pattern of arachin (solid line) and conarachin (dotted line) fractions of the peanut proteins.

## Discussion

- Mr. Donald M. Stockwell: In Dieckert's non-aqueous medium, fractionation of particles is shown; how is fractionation of particles in water media shown?
- Dr. Aaron M. Altschul: By separation by means of differential centrifugation before dissolving the proteins.
- Dr. Pierre Grabar: The use of a non-aqueous medium is ingenious; it may avoid hydrolysis by enzymes. I would like to hear more about it.
- Dr. Aaron M. Altschul: This technique did not seem to affect the solubility of the protein, nor certain enzyme activities.
- Dr. Joseph J. Rackis: I have several questions on the nature of extraction and the nature of the chromatography. Since soy and peanut have similar properties, can it be predicted where certain proteins will come out?
- Dr. Aaron M. Altschul: Our concern with the extraction was to be sure that no artifacts were being created. Several types of chromatography, electrophoresis, etc., should be tried. There is still the question whether or not you can really sort out proteins in this manner. There are three general groups of proteins: (1) does not stick on DEAE, (2) one sticks pretty hard (our group IV), and (3) intermediate groups. Varner's pea globulin seems to correspond to our group IV.

## THE PROTEINS OF COTTONSEED PIGMENT GLANDS

W. H. Martinez and L. C. Berardi

Southern Utilization Research and Development Division  
New Orleans, Louisiana

Cottonseed is unique among the oilseeds in that it contains certain discrete glands dispersed throughout the seed embryo which contain the polyphenolic pigment called gossypol (1). If a cottonseed is observed in a cross-sectional view, the pigment glands may be noted as readily visible black dots distributed throughout the seed kernel.

These globoids can be physically removed intact from the surrounding tissue. The glands may range in size from 100-400  $\mu$ , and contain 20-30% gossypol and 1-2% nitrogen.

The glands used in this experiment were obtained by the gland flotation process (2), that is, comminution of the dehulled seed and separation of the glands by a density gradient nonpolar solvent system. Though the glands are resistant to mechanical rupture, water or any polar solvent will rapidly disperse the contents of the glands.

The procedure used to fractionate the glands is schematically outlined in Diagram I. Successive extraction of the pigment glands with water and acetone removed essentially all the gossypol and produced a fraction (CC) which can be considered the gland wall fraction. This material (fraction CC) was further fractionated by extraction with 50% ethanol or with 1.5 M NaCl in 50% ethanol.

Each of the isolated materials was colored, due to the presence of gossypol, and could be shown on hydrolysis and analysis for amino acids to contain protein.



Microscopic examination of the residual gland walls (fraction CC) showed that the walls maintained their globular shape throughout the extraction procedure and were, for the most part, contaminated with small amounts of adhering parenchyma tissue. It therefore became necessary to determine if the proteinaceous materials found in the various fractions were components of the adhering tissue or the gland wall.

For this purpose, cold hexane extracted glanded cottonseed flakes were sieved through a 325 mesh screen. This procedure removed the hull particles and most of the pigment glands. The sieved material appeared to be primarily composed of the small globoids which could be noted, by microscopic inspection, in the cotyledonous parenchyma tissue surrounding the pigment glands. The sieved material was extracted in the same manner presented in Diagram I., e.g., successive extraction of the sieved cottonseed flakes with water and acetone followed by extraction of portions of the residue with 50% ethanol and 1.5 M NaCl in 50% ethanol.

No significant quantity of material was isolated from the sieved flakes by the acetone or 50% ethanol extraction procedures.

The material isolated by water extraction of the flakes (fraction H-0) represented 15% of the material by weight. An additional 20% by weight (fraction M 1.5) was extracted from the water, acetone residue by the alcohol-salt solvent. Each fraction contained minor amounts of gossypol due to the presence in the flakes of some gossypol from broken pigment glands.

The amino acid analyses of the fractions are recorded in Table I. Since the fractions varied widely in protein and nonprotein nitrogen contents, the data are presented as amino acid ratios, that is, the molar

ratio of each amino acid to the histidine content of the particular fraction. For purposes of ease of interpretation and presentation, each of the ratios was multiplied by 3. Included in the table, for purposes of comparison, is the amino acid analysis of an 80% ethanol extracted glandless cottonseed (CM72-A) calculated in the same manner. This pattern or profile represents the typical amino acid pattern which is obtained with whole cottonseed.

All fractions were hydrolyzed for 17 hours at 110° C. in ampules sealed under nitrogen at a ratio of 1 mg. of material per ml. of 6N hydrochloric acid and analyzed by means of the automatic Moore and Stein procedure (3).

The constant for glucosamine was established by the same procedure used for amino acids. Glucosamine appears in the standard Moore and Stein short column procedure at approximately 68 to 72 minutes running time. The peak for glucosamine coincides with that of tyrosine in the standard long column procedure for acidic and neutral amino acids and would, therefore, interfere with the tyrosine determination if present in any significant concentration.

The profile of each of the pigment gland fractions differs from that of the sieved flakes fractions, from that of the whole seed and from each other except fractions AA and E-0.

A number of factors characterize the profile of fraction AA. A small, immeasurable peak for hydroxyproline is consistently present. Glucosamine is present in almost equal ratio with lysine. Aspartic acid and alanine are present in equal ratio and in greater proportion than glutamic acid or glycine. Proline is present in unusually high proportion. All of these

characteristics differ from ratios found in the other fractions. They also, in part, suggest a certain relationship with those characteristics noted in connective and wall tissues, that is the presence of hydroxyproline and glucosamine and the high proline and alanine content (4).

Fraction BB is characterized by a high proportion of lysine, arginine, alanine, and glycine and a very low proportion of glutamic acid, cystine and methionine. This presents a unique amino acid profile.

The profile of fraction CC differs markedly from that of the whole cottonseed (72-A) in the ratios of lysine to arginine, aspartic acid to glutamic acid and isoleucine to leucine. This fraction, therefore, cannot primarily consist of the same proteinaceous materials that are found in the whole seed.

In fraction E-0 hydroxyproline is again noted as well as the other major characteristics specified for fraction AA; however, glutamic acid is present in even lower proportion than proline and a new unknown peak appears between leucine and tyrosine in the long column procedure. The concentration of the unknown peak, calculated with the leucine constant, appears to be in equal ratio with glucosamine. The fact that a fraction can be extracted from the gland wall residue which has a very similar composition to that proteinaceous material isolated by rupture of the glands with water, again suggests that this material is in some way associated with the gland wall. This concept is further enhanced by the fact that the amino acid profile of the water extract of the parenchyma tissue (H-0) in no way resembles that of fraction AA and E-0.



The major differences between fractions E-1.5 and M-1.5 occur in the ratios between arginine, aspartic and glutamic acids present in each of these fractions. Solubility differences also exist. Fraction M-1.5 is completely soluble in 0.01 M acetic acid; however, 50% of E-1.5 is insoluble. This may be due in part to the gossypol present in this fraction, none of which chromatographed as free gossypol (5). Examination of the amino acid composition of the soluble and insoluble portions of fraction E-1.5 indicates that the material extracted from fraction CC with 1.5 M NaCl in 50% ethanol (E-1.5) is probably a mixture of the material soluble in 50% ethanol (E-0) and the material soluble in 1.5 M NaCl in 50% ethanol from the parenchyma tissue which adheres to the pigment gland wall.

Electrophoresis of each of the fractions, except CC and 72-A, was performed in a vertical electrophoresis apparatus using acrylamide gel (6) and a TRIS (0.25 mol.), EDTA (0.0105 mol.), Boric acid (0.0375 mol.) buffer at pH 8.9 (7). After differences in the staining ability of amino black 10B and Nigrosine were noted, a 1% solution of Nigrosine in a methanol, water, acetic acid (10:10:1) solvent was used for staining. Figure 1 illustrates the differences in staining ability obtained with the same 0.5% solution of H-0. Whether the differences noted are due to actual differences in the dye-binding ability of the proteins or the greater insolubility of the Nigrosine dye-protein complex is not known.

Figure 2 illustrates the number of protein components in the water and alcohol-salt soluble fractions of the glanded (H-0 and M-1.5) and glandless cottonseed.



Electrophoresis of solutions of pigment gland fractions AA, BB and E-0 with concentrations as high as 3% did not produce even faintly discernible protein bands. This could possibly be due to the solubility of the protein-dye complex in the wash solution or the inability of the material to enter the gel. The highly colored solutions were, however, always noted to enter the gel.

Fraction E-1.5 gave only a single protein staining band which moved very rapidly with the front. This band was readily observed before staining because of the intense yellow color which would indicate the presence of gossypol. Electrophoresis of the soluble and insoluble portions of E-1.5 showed that this band was primarily concentrated in the insoluble portion.

It therefore appears that the pigment glands of cottonseed contain proteinaceous materials which are unique in amino acid pattern and electrophoretic response. Whether these proteins are associated with the gland wall or constitute perhaps a part of a gossypol-protein complex inside the gland remains to be elucidated.

#### References

1. Boatner, C. H., "Cottonseed and Cottonseed Products," A. E. Bailey, ed., p213, Interscience, New York, 1948.
2. Boatner, C. H., Hall, C. M., Oil and Soap, 23, 123 (1946).
3. Spackman, D. H., Stein, W. H., Moore, S., Anal. Chem., 30, 1190 (1958).
4. Salton, M. R. J., "Microbial Cell Walls," Wiley and Sons, New York, 1960.
5. Schramm, G., Benedict, J. H., J. Am. Oil Chemists' Soc., 35, 371 (1958).
6. Raymond, S., Wang, Y. J., Anal. Biochem., 1, 391 (1960).
7. Aronsson, T., Gronwall, A., Scand. J. Clin. Lab. Invest., 9, 338 (1957).

Diagram I. Extraction Procedure

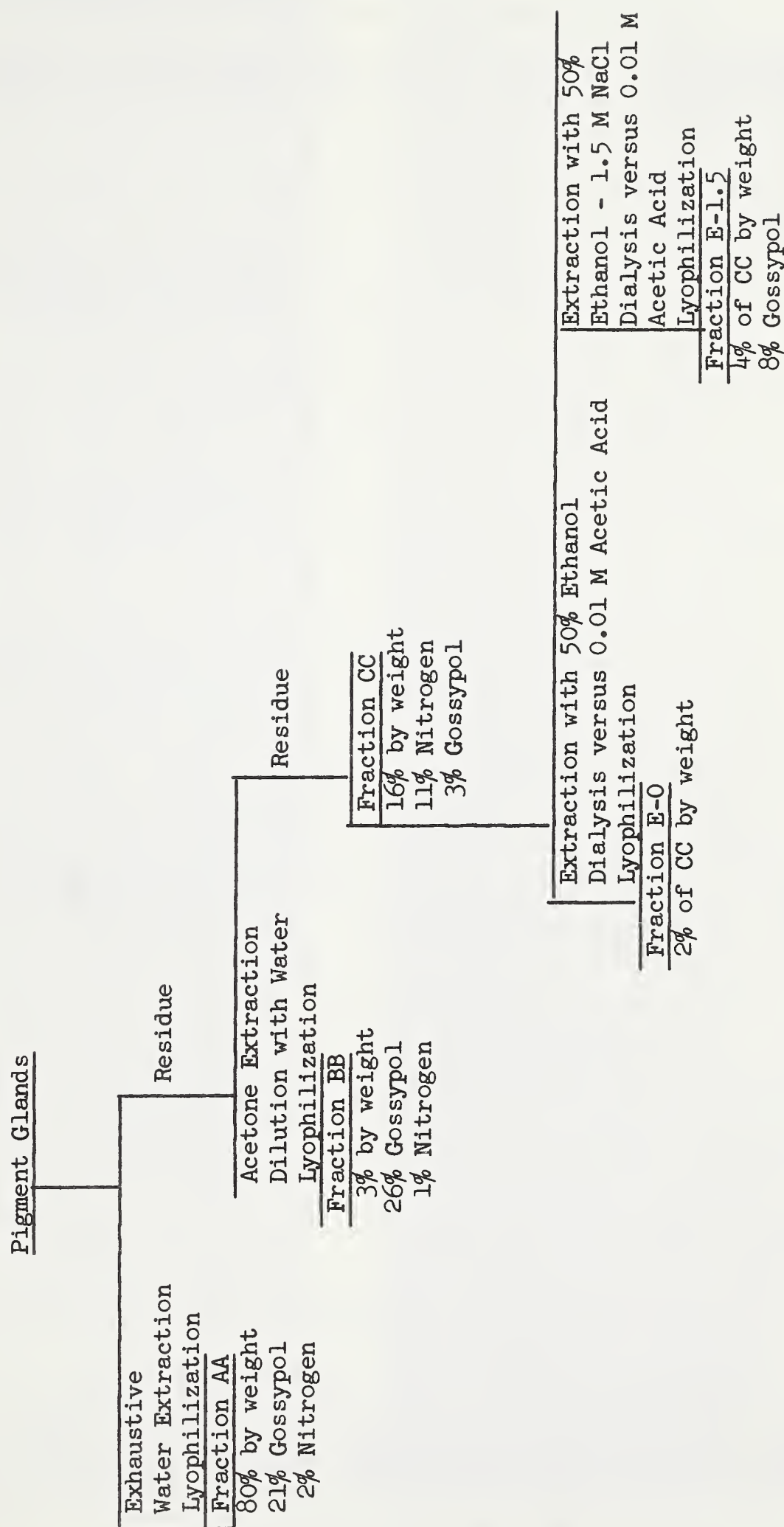


Table I. Amino Acid Profiles<sup>1/</sup> of Fractions

Amino Acid	AA <sup>2/</sup>	H-O <sup>2/</sup>	BB <sup>2/</sup>	CC <sup>2/</sup>	72-A <sup>2/</sup>	E-O <sup>2/</sup>	E-1.5 <sup>2/</sup>	M-1.5 <sup>2/</sup>
Glucosamine	8	0.5	2	0.2	--	11	1	0.4
Lysine	11	10	17	8	5	12	6	5
Histidine	3	3	3	3	3	3	3	3
Arginine	20	21	19	9	10	26	13	15
Aspartic Acid	33	17	13	13	11	43	12	9
Threonine	23	5	8	7	5	36	6	2
Serine	23	7	6	8	6	36	8	5
Glutamic Acid	25	38	5	17	22	22	19	26
Proline	19	8	4	7	5	27	5	4
Glycine	28	11	32	12	9	32	11	10
Alanine	33	7	20	11	7	42	9	4
$\frac{1}{2}$ Cystine	10	9	0.6	1	1	22	6	6
Valine	11	5	10	8	7	12	4	3
Methionine	2	2	0.2	2	1	5	2	3
Isoleucine	14	3	8	5	5	21	4	2
Leucine	28	6	9	10	8	43	8	5
Unknown	0	0	0	0	0	11	1	0
Tyrosine	--	4	4	4	3	--	3	3
Phenylalanine	--	4	8	5	5	8	3	7
% by weight of fraction accounted for by amino acid analysis	5	26	2	60	50	16	33	64

<sup>1/</sup> Analyses are reported as amino acid ratios. See text for calculation.

<sup>2/</sup> AA - Water-dispersible fraction of Pigment Glands; H-O - Water-soluble fraction of Cottonseed Meal; BB - Acetone-soluble fraction of water-extracted Pigment Glands; CC - Residue from water and acetone extraction of Pigment Glands; 72-A - 80% Ethanol-extracted Glandless Cottonseed Meal; E-O - 50% Ethanol-soluble fraction of CC; E-1.5 - 50% Ethanol - 1.5 M NaCl-soluble fraction of CC; M-1.5 - 50% Ethanol - 1.5 M NaCl-soluble fraction of water-extracted Cottonseed Meal.

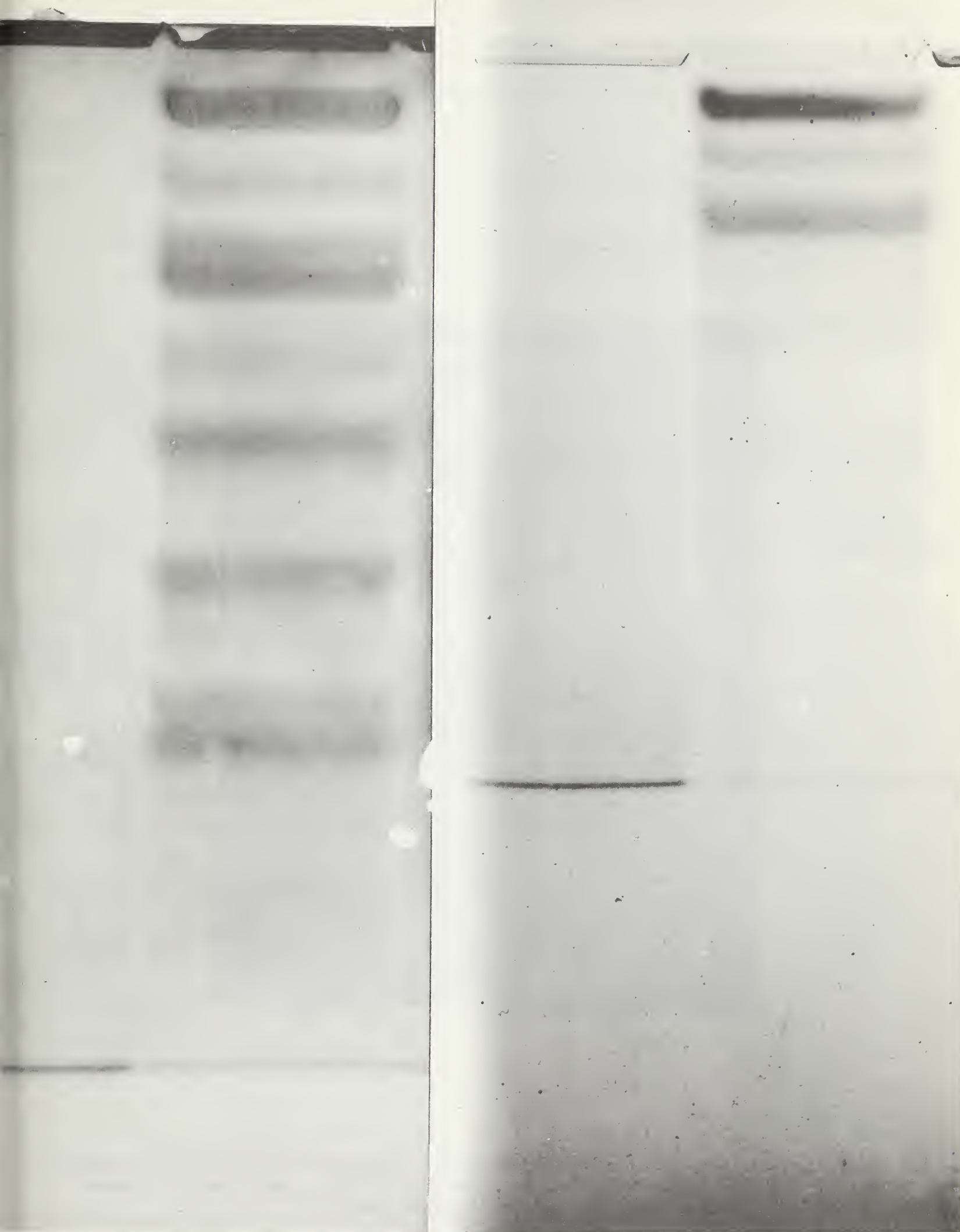


FIGURE 1. From left to right: Fraction E-1.5P, Fraction H-O stained with Nigrosine; Fraction E-1.5P, Fraction H-O stained with amido black 10B



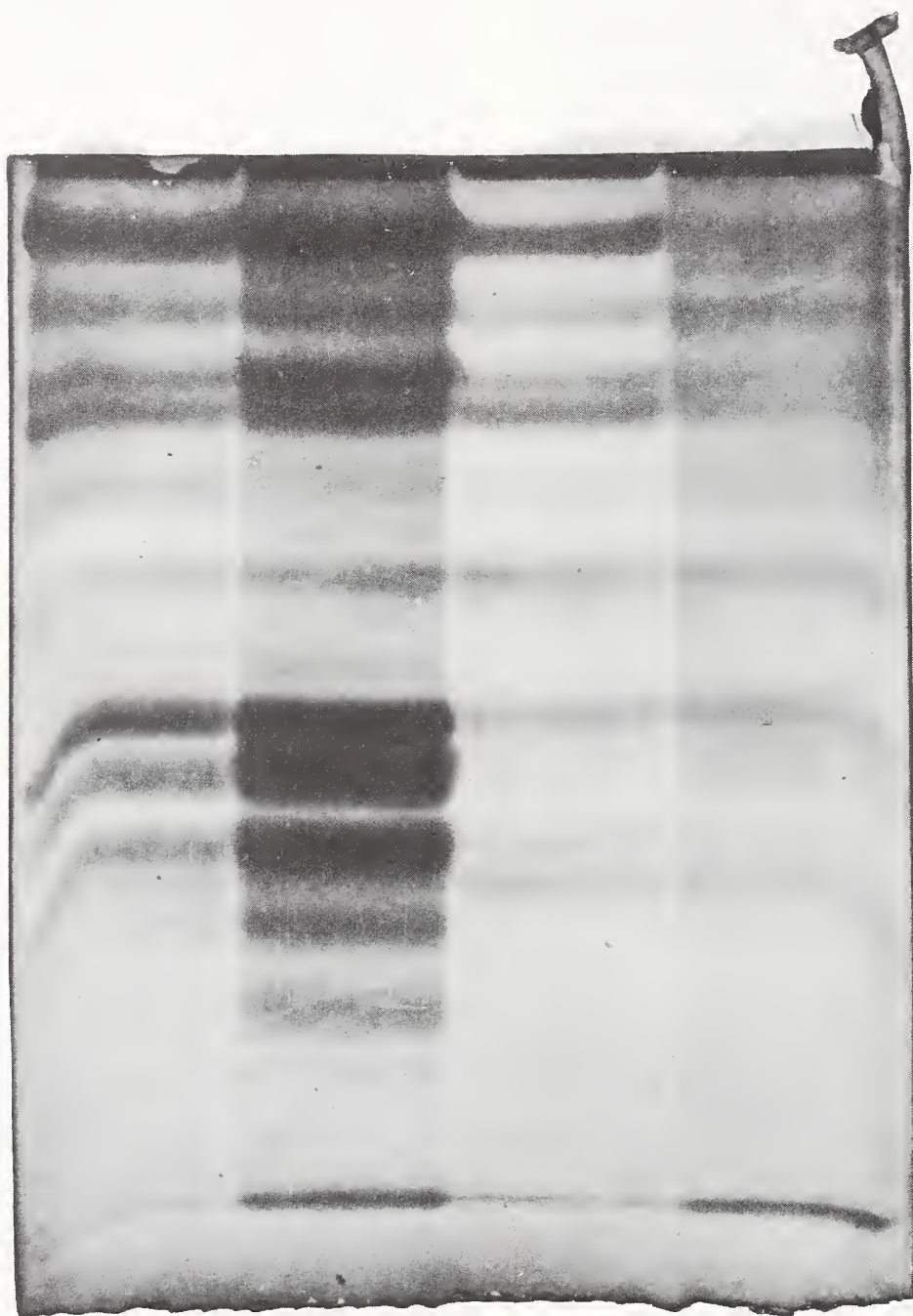


FIGURE 2. From left to right: Alcohol - salt soluble fraction of glandless cottonseed meal; Fraction M-1.5; Fraction H-0; Water extract of glandless cottonseed meal.

## Discussion

- Dr. Virginia R. Williams: How can you be sure the glucosamine is part of the protein and not part of the carbohydrate?
- Mrs. Wilda H. Martinez: I'm not saying that it is part of the protein.
- Dr. Pierre Grabar: Have you tried substituting trichloroacetic acid for acetic?
- Mrs. Wilda H. Martinez: Comment: (I do not recall the particular question attributed to Dr. Grabar. It does not seem to be appropriate to the talk. I do remember Dr. Grabar noting that he too on occasion was unable to obtain protein stains on agar electrophoresis. Perhaps, it is in this connection that he was suggesting that the protein be precipitated in the gel with trichloroacetic acid. This has not been tried. Dr. Gross also noted that the position of the unknown peak on the long column of the Moore and Stein procedure seemed to indicate that it might be a dipeptide and that extended hydrolysis periods should be tried.)

# LIPID-PROTEIN PARTICLES IN THE COTTONSEED

L. Y. Yatsu  
Southern Utilization Research and Development Division  
New Orleans, Louisiana

## ABSTRACT

A cytological investigation was conducted as a prelude to further biochemical studies on the lipid metabolism of the cottonseed. An attempt to define the exact location of lipids in the cottonseed was conducted at two levels: (1) the light microscope level, and (2) the electron microscope level.

Light Microscopy. Light microscopy studies of the cottonseed failed to yield evidence of readily discernible, "free" lipids within the cells. Numerous spherical bodies were seen in the parenchyma cells of the cotyledon and it was theorized that these spherical bodies were the site of oil storage within the cells. An attempt, therefore, was undertaken to isolate them.

The conventional methods of isolation of subcellular particles proved inadequate for isolating the spherical bodies seen in the cells. Cottonseeds ground in a buffered, isotonic medium yielded "free" oil; thus the integrity of the spherical bodies was lost. Integrity of the particles was maintained momentarily when hypertonic grinding mediums were used, but this, too, proved inadequate.

A novel technique was devised to permit isolation of the spherical bodies. Cottonseed was ground in a glycerol-tannic acid solution to "tan" a postulated membrane around the particles. Subcellular particles obtained

in this manner were not affected by water and could be isolated by differential centrifugation. The spherical bodies obtained resembled the so-called aleurone grains which have been reported in other seeds.

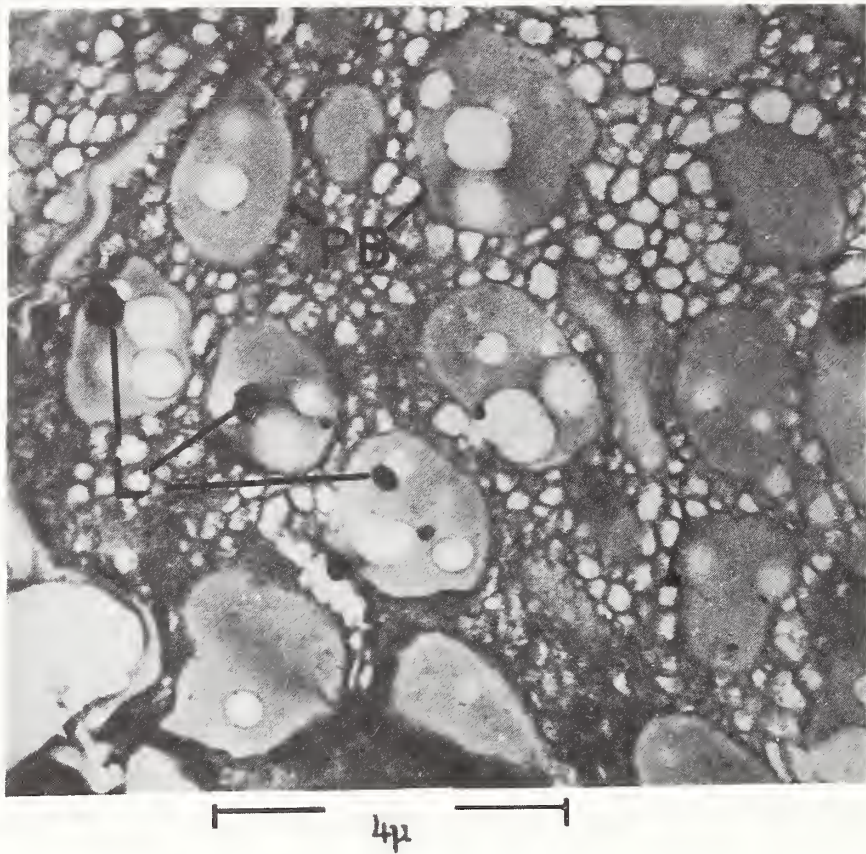
Staining with an alcoholic solution of Sudan 4 demonstrated the presence of lipids within the spherical bodies. Ethyl ether extraction of a lyophilized preparation of spherical bodies yielded on the order of 25% to 28% lipids.

Electron Microscopy. The cotyledon parenchyma cells contained numerous spherical bodies which were stained lightly by the osmium. The spherical bodies were interspersed within a foam-like reticulum. Most of the in situ spherical bodies contained holes, many of which were filled with highly osmiophilic substances. Under higher magnification the osmiophilic areas were found to be composed of an extremely fine reticulum, thought to be the site of oil storage.

Many areas along the interbody network were also darkly stained by the osmium. The darkly stained areas under higher magnification were also shown to be a minute reticulum which was also interpreted to contain oil.

The cellular composition of the axial tissue was completely different from the cotyledonary tissue. There was a very high degree of organization in the tissue taken from the radicle of the cottonseed as contrasted to the relatively simple appearance of the cotyledonary tissue. Certain of the cellular particles in the radicle were reminiscent of the aleurone grains, others resembled the endoplasmic reticulum, but none of the particles could be identified as mitochondria.





Cross section of a cotyledonary parenchyma cell  
of cottonseed.

## Discussion

Dr. Te May Ching:

We have found both lipid-droplets and protein bodies in germinating Douglas fir seed endosperm by electron-micrography. The protein bodies could be isolated in 0.5 M mannitol-phosphate buffer (pH 6.8, 0.1 M) at 900 X<sub>g</sub> for 10 minutes. The protein bodies contain 20-25% lipids and 60-70% protein. The lipid-droplets remained on top of the medium after centrifugation and were almost completely soluble in chloroform-methanol (3=1 by vol.)

Dr. Donald N. Duvick:

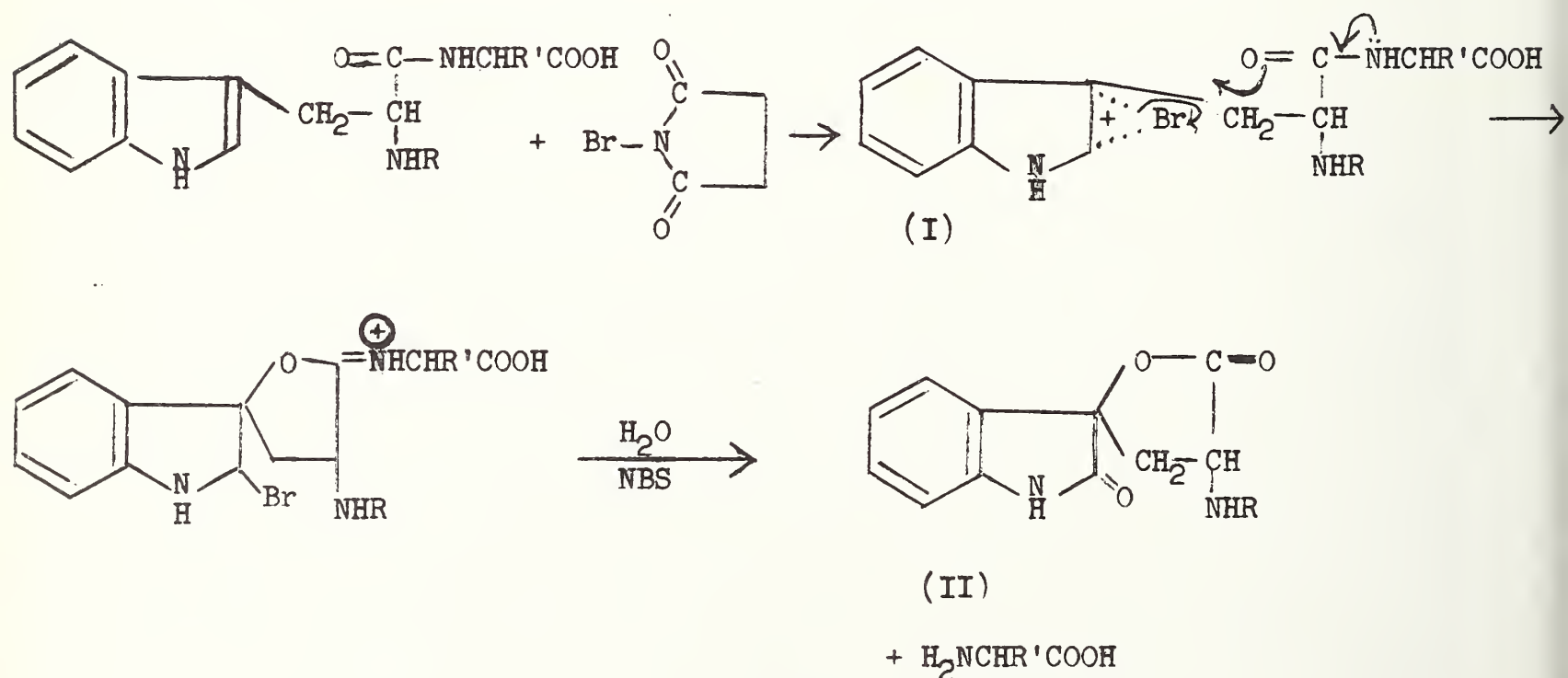
The pictures remind me of aleurone grains, however, the contents of these are quite different.

# NONENZYMATIC CLEAVAGE OF PEPTIDE BONDS

Erhard Gross  
National Institutes of Health  
National Institute of Arthritis and Metabolic Diseases  
Laboratory of Chemistry  
Bethesda, Md.

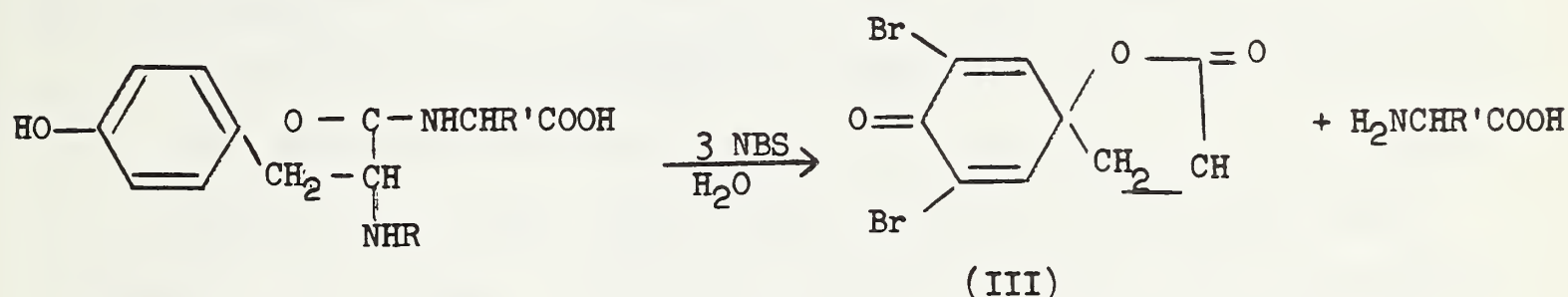
Amino acids with easily accessible additional functional groups can readily be subjected to chemical modification. If such a modification occurs in a peptide at a suitably located carbon atom, e.g. at the  $\gamma$ -carbon atom, the carbonyl function of the same amino acid is able to participate in the reaction, and via formation of an iminolactone and subsequent facile hydrolysis, a peptide bond can easily be broken.

Thus tryptophan peptides react with N-bromosuccinimide (NBS) (1) and other N-halocompounds to form the 2,3-dioxindole-spirolactone (II) and cleave the peptide bond. Intermediate product is the bromonium ion (I) the result of the attack of the positive bromine of NBS at the indole nucleus of tryptophan.



Gramicidin A a polypeptide antibiotic, like many other peptides and proteins, is not attacked by enzymes. It contains 45% tryptophan and has been cleaved successfully with NBS and N-bromoacetamide (2).

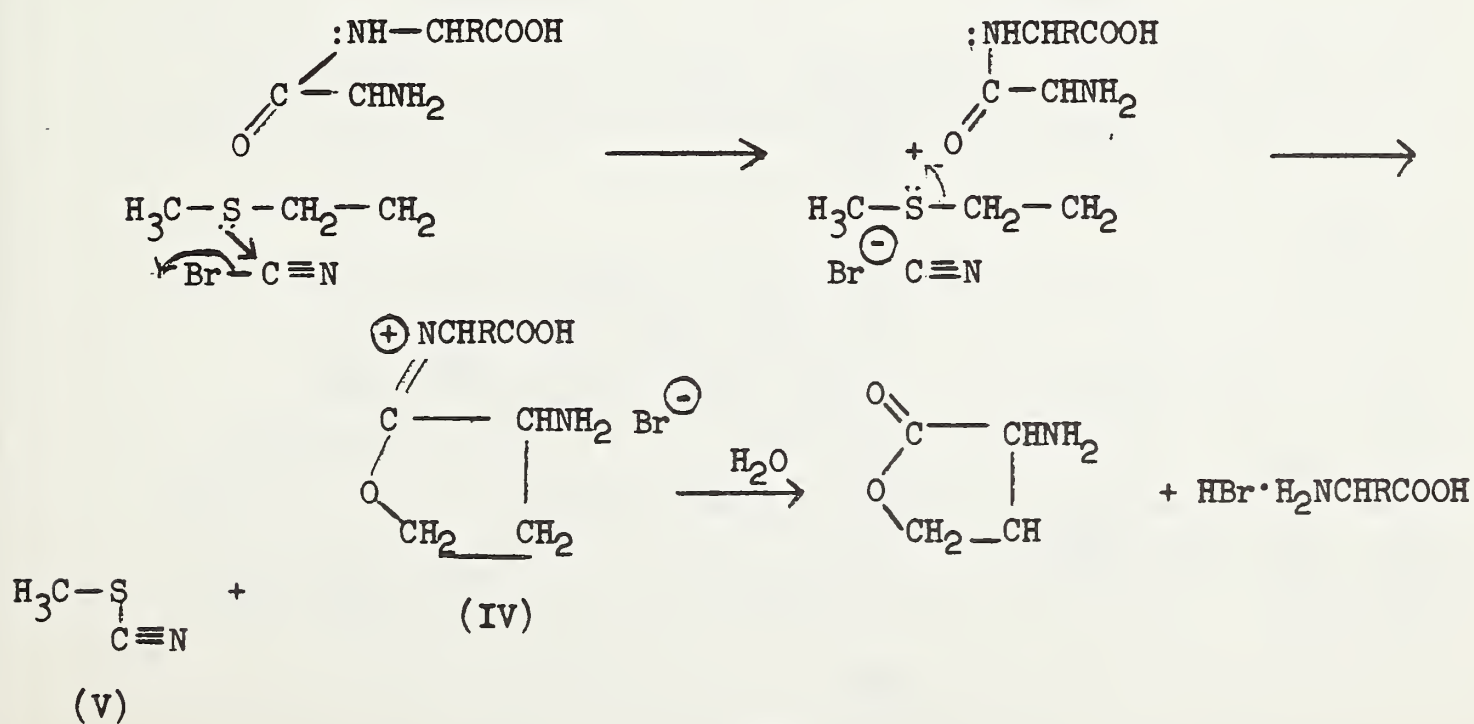
Tyrosine (3) is also susceptible to attack by N-halocompounds and forms a dibromodienone spirolactone (III) to split the peptide bond.



The rate of reaction for tyrosine is low enough that one tryptophan peptide bond in glucagon (1,4) could be cleaved in the presence of tyrosine peptide bonds, these not having been affected.

Other oxidizing reagents are able to distinguish between these two amino acids. Under mild reaction conditions, sodium iodate or -periodate (2), e.g., will react with tryptophan only and not with tyrosine.

A reagent of highly improved selectivity is cyanogen bromide which reacts with methionine (5) only, converting it to homoserine lactone (IV)





and methylthiocyanate (V) and releasing the next following amino acid.

Bovine pancreatic ribonuclease with four residues of methionine and one of the first proteins to have its primary structure reported appeared to be a good example for a first application of the method.

All four methionyl peptide bonds were cleaved at room temperature in 0.1 N HCl during a 24 hour reaction period (6). The results of this investigation have led to a revision of the sequence for positions 11 through 18 (6,7,8) that had earlier been reported for the enzyme (9).

Other enzymes, e.g. trypsin, chymotrypsin, pepsin, are presently being investigated for their possible nonenzymatic cleavage with cyanogen bromide.

The mild reaction conditions under which "chemical peptidases" like BrCN work and the relatively large segments that result from proteins by breaking only a few peptide bonds, will perhaps make possible the isolation of enzyme fragments which still carry part or full activity.

#### References

1. Patchornik, A., Lawson, W. B., Gross, E., and Witkop, B.: J. Am. Chem. Soc. 82, 5923, 1960.
2. Preliminary data in: "Nonenzymatic Methods for the Preferential and Selective Cleavage and Modification of Proteins," by B. Witkop: Adv. Protein Chem. 16, 221, 1960.
3. Schmir, G. L., Cohen, L. A., and Witkop, B.: J. Am. Chem. Soc. 21, 2228, 1959.
4. Bromer, W. W., Staub, A., Diller, E. R., Bird, H. L., Sinn, L. G., and Behrens, O. K.: J. Am. Chem. Soc. 79, 2794, 1957.
5. Gross, E. and Witkop, B.: J. Am. Chem. Soc. 83, 1510, 1961.
6. Gross, E. and Witkop, B.: J. Biol. Chem., 237, 1856, 1962.
7. Smyth, D. G., Stein, W. H., and Moore, S.: J. Biol. Chem. 237, 1845, 1962.
8. Potts, J. T., Berger, A., Cooke, J., and Anfinsen, C. B.: J. Biol. Chem. 237, 1851, 1962.
9. Hirs, C. H. W., Moore, S., and Stein, W. H.: J. Biol. Chem. 235, 633, 1960.

## Discussion

- Dr. Aaron M. Altschul: Would you comment on whether native proteins will react?
- Dr. Erhard Gross: No general statement can be made at this time. In the case of ribonuclease acid denaturation had been required. For other proteins we have to obtain more information.
- Dr. M. L. Anson: The study of the reactions of protein groups without the splitting of peptide bonds is still a neglected field.
- One reaction of some interest in connection with the work of Gross is the oxidation of SH to -S-S- by  $I_2$  in 1 M K I at 0° C. The reaction does not go beyond -S-S- and there is no reaction of the  $I_2$  with tyrosine or tryptophan.
- Dr. Mark A. Stahmann: Do you have more information on Chymotrypsin? Must you cleave the S-S bonds before the cyanogen bromide reaction?
- Dr. Erhard Gross: Yes, this is under investigation now.

## CYANATE AS A REAGENT IN PROTEIN CHEMISTRY

George R. Stark  
The Rockefeller Institute  
New York, N. Y.

In 1828, Wöhler published his observation that urea could be formed by heating ammonium cyanate (1). Almost 70 years later, Walker and Hambly discovered that the reaction is reversible, i.e., that aqueous solutions of urea can give rise to small amounts of cyanate (2). Since cyanate ion reacts rapidly with primary amines ( $\text{RNH}_3^+ + ^-\text{NCO} \longrightarrow \text{RNH} \overset{\text{O}}{\underset{\text{O}}{\parallel}} \text{C} \text{NH}_2$ ) and even more rapidly with sulfhydryl groups ( $\text{RSH} + ^-\text{NCO} + \text{H}^+ \longrightarrow \text{RS} \overset{\text{O}}{\underset{\text{O}}{\parallel}} \text{C} \text{NH}_2$ ), the concentrated urea solutions often used as denaturants for proteins may give rise to chemical as well as physical changes (3). Fortunately, the formation of cyanate from urea is slow and urea solutions can be kept essentially free of cyanate if precautions are taken (4).

Cyanate is an unusual reagent in that it reacts with charged rather than uncharged amino groups (5). This property enables one to compare the reactivities of  $\epsilon\text{-NH}_2$  groups in proteins at neutral pH values rather than at the more alkaline values required for reaction with most other reagents. Completely carbamylated proteins can be readily prepared using KNCN in aqueous urea or guanidinium chloride (6). Such derivatives may be useful in achieving separations of mixtures of basic proteins since the derivatives have fewer positive charges. A serious limitation to such use is that the carbamyl group cannot be easily removed.

$$\begin{array}{c} \text{O} \quad \quad \quad \text{O} \\ \parallel \quad \quad \parallel \\ (\text{NH}_2 - \text{C} - \text{NHCHR} - \text{C} - ) \longrightarrow \text{NH} - \text{C} - \\ | \quad \quad \quad | \\ \text{O} = \text{C} - \text{CH} - \text{NH} \\ | \\ \text{R} \end{array}$$

## References

- ## Discussion

Could the temperature for the hydantoin formation be lowered?

Lowered temperature was unsuccessful.

An interesting application would be the use of cyanate to determine free  $\epsilon$ -amino groups in foodstuffs and thus reflect on the available lysine after processing.



INTERACTION OF BROM THYMOL BLUE WITH PROTEINS  
AND ITS RELATION TO THEIR CONFORMATION

Eraldo Antonini  
Istituto di Chimica Biologica  
Universita di Roma  
Rome, Italy

The sulfonphthalein pH indicator brom thymol blue combines reversibly with proteins, the pK of the bound dye being increased (1) (about 1 pH unit in the case of hemoglobin and serum albumin). The affinity for the dye is a highly specific property of the protein and depends on its conformational state. The rate of combination of the dye with proteins is rapid and it also is highly specific for the protein and its conformation. This is illustrated by studies on the combination of brom thymol blue with hemoglobin and its derivatives, where the half time of the reaction varies between 10 and 100 milliseconds for a concentration of dye and protein of  $\sim 10^{-4}$  M.

Oxy-, carbonmonoxi-, and ferri-hemoglobin which are all isomorphous in the crystal show the same rate; deoxygenated hemoglobin, which is known to have a different conformation (2), shows a rate 3 to 4 times greater. Also the affinity of deoxyhemoglobin for the dye is greater than that of oxyhemoglobin and this is reflected by the effect of the dye on the oxygen equilibrium of the protein.

The apoprotein, globin, reacts vastly faster than any of the hemoglobins. The characteristic difference of reactivity toward brom thymole blue shown by the oxygenated and deoxygenated derivatives of normal hemoglobin is absent in myoglobin and in those modified forms of hemoglobin (3) which have simple hyperbolic oxygen equilibrium curves.

It would appear from these results that the study of the interaction of brom thymol blue with proteins might give significant information on conformational changes which they may undergo, particularly in the case of enzymes.

### References

1. Antonini, E., Wyman, J., Moretti, R., and Rossi Fanelli, A., Biochim. Biophys. Acta - in the press.
2. Perutz, M. F. Proteins and Nucleic acids - Elsevier 1962 Amsterdam.
3. Antonini, E., Wyman, J., Zito, R., Rossi Fanelli, A., Caputo, A., - J. Biol. Chem. 236, PC 60, 1961.

### Discussion

Dr. Pierre Grabar:

The reaction of an enzyme with its substrate at one site and with its antibody at another is an example of this phenomenon. If the reacting groups are close, steric hinderance of the reaction with the substrate may occur. I have not made much study of the effect of the reactivity of one site by a reaction having occurred at another site. In the case of enzyme reactions, perhaps the pH optimum is changed.

Dr. Abraham Marcus:

Inhibition of a reaction by end-products may be studied by this method.

Dr. Mark A. Stahmann:

Can electron transfer occur along a peptide chain just as it does along conjugated double bonds?

OPTICAL ROTATION IN RELATION TO PROTEIN STRUCTURE  
Observations on Plant Proteins\*

B. Jirgensons  
The University of Texas  
M. D. Anderson Hospital and Tumor Institute  
Department of Biochemistry  
Houston, Texas

Introduction

It is now well known that the optical activity of a protein depends not only on the presence of the asymmetric carbon atoms in the polypeptide chains but also on the chain configuration. The optical rotatory power of a protein changes on denaturation, i.e. without any chemical change at the asymmetric carbon atoms of the polypeptide chain. Nowadays, the term "configuration" is retained for the asymmetry of the carbon atoms in each of the amino acid residues, whereas the geometry of the polypeptide chain as a whole is termed "conformation". The crucial test regarding the usefulness of the optical rotation method for the study of protein structure and conformation was that of confronting the results of this method with the most straightforward X-ray structural analysis. Both methods were compared recently in the case of the muscle protein myoglobin. The high resolution X-ray structural analysis of Kendrew et al, on myoglobin crystals (1) and the optical rotation studies of Beychok and Blout (2) and Urnes et al, (3) on the solutions of this protein have shown conclusively that about 70% of the polypeptide chain of myoglobin is in the  $\alpha$ -helical conformation, and that the helical chain is folded into a compact body.

---

\* Supported in part by grants from the National Cancer Society, National Institutes of Health, U. S. Public Health Service (Grant C-1785), the Robert A. Welch Foundation, Houston (Grant G-051), and American Cancer Society (Grants P-262 and P-263).



One of the fundamental magnitudes in the optical rotation studies is the specific rotation  $[\alpha]$ , which is defined as

$$[\alpha] = \alpha \cdot 100 / c \cdot l,$$

where  $\alpha$  is the observed rotation of the plane of polarization in angular degrees  $c$  concentration in grams per 100 cc, and  $l$  the length of the solution layer in decimeters. Although the specific rotation appears as a quite useful characteristic for some structural changes in proteins, much more information on structure and conformation is obtainable by the study of optical rotatory dispersion, i.e. the dependence of the specific rotation on the wave length of the light. The optical activity increases with decreasing wave length, and, if the measurements are made far enough from the absorption bands, the relationship can be expressed by either the Drude rule or the Moffitt equation. The latter is now known to be more useful for proteins than the former, and the theoretical background of the whole matter is discussed in recent reviews (4, 5). For practical convenience and accuracy, the Drude rule was rearranged by Yang and Doty (6) in the following form:

$$[\alpha] \cdot \lambda^2 = [\alpha] \cdot \lambda_c^2 + K,$$

where  $\lambda$  is the wavelength and  $K$  and  $\lambda_c$  are constants. For structural considerations, the constant  $\lambda_c$  appeared to be of great interest, and the  $\lambda_c$  values have been determined now for over 50 proteins (4, 5, 7, 8). To obtain  $\lambda_c$ , the product  $[\alpha] \cdot \lambda^2$  is plotted against  $[\alpha]$ , and  $\lambda_c$  is obtained from the slope of the straight line. The Moffitt equation has several more parameters, viz.:

$$[R'] = [\alpha] \cdot 3M/100 (n^2 + 2) = a_0 \lambda_0^2 / (\lambda^2 - \lambda_0^2) + b_0 \lambda_0^4 / (\lambda^2 - \lambda_0^2)^2,$$



in which  $[R']$  is the corrected specific residual rotation,  $M$  the mean molecular weight of the amino acid residues,  $n$  refractive index of the solvent, and  $a_0$ ,  $b_0$  and  $\lambda_0$  adjustable parameters (9). It has been found that  $\lambda_0 = 212 \text{ m}\mu$  yields satisfactory results in the case of proteins and synthetic polyamino acids (9). Using this value, and having the other experimental data, one can plot the product  $[R'] \cdot (\lambda^2 - 212^2)$  against  $212^4/(\lambda^2 - 212^2)$ , and this plot yields straight lines. Again, the slope constant  $b_0$  is the one most desired, since it can be correlated to the conformation of the polypeptide chains. If the mean residual weight and refractive index corrections are not available, a somewhat less accurate  $b_0$  can be obtained by plotting  $[\alpha] \cdot (\lambda^2 - 212^2)$  against  $212^4/(\lambda^2 - 212^2)$  or  $1/(\lambda^2 - 212^2)$ .

The purpose of the present paper is to describe recent advances in the spectropolarimetry of proteins with a special reference to plant proteins.

#### Experimental Procedures

Recent achievements in the spectropolarimetry of proteins have been facilitated by the availability of improved instruments. Fig 1 shows a schematic sketch of the O. C. Rudolph photoelectric spectropolarimeter, now in use in many laboratories. Similar instruments have been used in this laboratory since 1957. The light beam from a light source enters, first, a monochromator which selects a narrow beam of a definite wave length. This beam then is reflected into the polarimeter, and the effect of the specimen on the plane of polarization is detected and measured with a phototube and sensitive photometer. The extinction point is measured indirectly, i.e. by approaching it from both sides. This procedure with the older instruments was performed manually by making photometer readings

after turning the analyzer prism a certain number of degrees off the approximate zero point, and by obtaining the extinction point by calculation from a series of readings. Since this is time consuming, more recently one of the prisms, usually the polarizer is rocked symmetrically by a motor and the extinction point obtained directly, after proper adjustment of the analyzer prism. The most efficient light sources are mercury lamps or xenon lamps, e.g. the Engelhard-Hanovia xenon-mercury arc source operated at 250 watts AC or DC at a proper stabilization of the current. This permits the optical activity measurements at wave lengths of 578, 546, 492, 436, 405, 365, 334, 313.1, 296.7 m $\mu$ , and even shorter wave lengths, if the specimen is not too absorbing. A plain xenon white light source is usable at any wave length in the visible and near ultraviolet part of the spectrum, but it yields reliable results only if operated with narrow monochromator slit settings of 0.1-0.3mm. Although wider slits produce higher intensity, the spectral purity at wider slits of 1-2mm is usually so deteriorated that the data become erroneous. The same is true, for any other white light sources, such as the zirconium lamps. The performance of the instrument, depends also on the quality of the prisms, lenses, and mirrors, on the sensitivity of the detector, and other factors. Quartz optics is used in the best instruments. It has been found recently that the exclusion of stray light is best achieved by using monochromators which have two dispersing prisms in series. If such an instrument is not available two single prism monochromators can be placed in series, as it was done in the laboratory of the author. A RCA 7200 phototube is one of the best sensing units, and the sensitivity can be boosted by applying high voltages

of 500-1000 volt across the dynodes. The checking of zero point by using the sample tube with solvent is important, especially regarding the end plates of the tube and its position. The best reproducibility is obtained by using cells with fixed fused silica end plates and placing the cell always in the same place in the trough. The accuracy and reproducibility depend on the stability of the light beam, on its intensity, on the monochromaticity of the polarized beam (absence of stray light) and on the performance of the detecting unit. The condition of the protein solution, of course, also is important, since maximum clear and colorless samples will permit the use of higher concentrations and greater layer thicknesses than solutions which absorb and scatter light strongly. In the work reported in this paper, the protein solutions were colorless (or slightly colored because of small amounts of colored impurities), the protein concentration ( $c$ ) was 0.1 - 4%, and the tube length ( $l$ ) was 1-10 cm. In most cases, the protein concentration was 0.5 - 1%, and cell length was 1 dm. The accuracy of the specific rotation values was 0.5 - 2 degrees, depending on the wave length; the accuracy of the dispersion constant  $\lambda_c$  was in the range of 1-5  $m\mu$ , and the accuracy of  $b_0$  was approximately  $\pm 5^\circ$ .

More recently, considerable progress has been made in the far ultraviolet spectropolarimetry (10, 11, 12), i.e. in the 180-300  $m\mu$  spectral range. The experimental difficulties in this range increase enormously, because of the tremendous increase of absorption of light with decreasing wave length. The protein concentration is reduced to 0.01 - 0.1 %, and the cell length (solution layer thickness) to 0.1-1 cm. Although the



rotatory power increases strongly with decreasing wave length, small angles of a small fraction of angular degree being measured results in a loss of accuracy. The ordinary 250 watt mercury-xenon lamp is useful only to about 220 m $\mu$ . At still lower wave lengths a 450 watt xenon source has yielded acceptable results (11).

### General Results

Protein solutions are levorotatory, and the numerical value of this levorotation usually increases upon disorganization of the native conformation. About a decade ago, this change of levorotation was known as the only characteristic of structural change. Since there was not a clear theoretical basis for such change, and since there were no obvious quantitative relationships, this approach was of little value in the study of protein structure. The situation became more hopeful after invoking the new theoretical considerations (4, 5, 6, 9, 13) and experimental data on optical rotatory dispersion (4, 5, 6, 7, 8). In 1956-57 it seemed that all native globular proteins were basically of the same  $\alpha$ -helical conformation with their  $\lambda_c$  values being about 230-280 m $\mu$ , and that the  $\lambda_c$  decreases to 210-220 m $\mu$  on denaturation, viz. disorganization of the native conformation (6). Soon afterwards, however, it was discovered that a group of native globular proteins (e.g. serum  $\gamma$ -globulin, pepsin, and soybean trypsin inhibitor) possess dispersion constants of 200-220 m $\mu$  (14, 15), and that there were even some others (e.g. the urinary Bence-Jones proteins) with  $\lambda_c$  values of 180-190 m $\mu$  (16). Also it was found then that the dispersion constants of these proteins increased slightly on denaturation. This indicated that the relationships were more complex than it was assumed



before. Accordingly, the author of this paper indicated that various globular proteins probably have a variety of conformations, and, regarding the optical rotatory properties, suggested to classify the proteins into three groups (7, 8, 15), as indicated in Table I.

Table I contains 36 examples, 12 in each group. Approximately 30 more proteins, up to now, have been studied by spectropolarimetric methods, and most of them belong to the Groups I and II. Plant proteins appear in all three Groups. There is little doubt that large segments of the polypeptide chains in the Group I proteins are in the  $\alpha$ -helical conformation, whereas the conformation of the other proteins is still controversial, as pointed out especially by Tanford et al (17). Since there are reasons to believe that these proteins are not disordered, attempts have been made to look for orders other than the  $\alpha$ -helix. These endeavors, however, have not been very successful.

New leads in correlating the rotatory dispersion data with conformation became available when the spectropolarimetric technique permitted to explore the Cotton effects in the far ultraviolet spectral range (10, 11, 12). It was found that the proteins which have high  $\lambda_c$  values, and strongly negative  $b_0$  constants, showed a negative Cotton effect with a minimum at 232-234 m $\mu$ , whereas the disordered proteins, and also those of Group III (Table I), exhibited smooth curves in the 220-300 m $\mu$  spectral region. This is illustrated in Fig. 2. The levorotation of the Group III proteins or e.g. of reduced and carboxymethylated serum albumin (which represents a fully disordered random chain) increases monotonously, according to a power function, with decreasing wave length (dotted curve), whereas the proteins with a high  $\lambda_c$  exhibit a minimum. At still lower wave lengths the levoro-

tation of these proteins decrease with decreasing wave length, and at a certain point the sign changes from minus to plus (Fig. 2). It was attempted then to correlate the depth of the minimum (or amplitude of the Cotton effect) with the  $\alpha$ -helix content of the proteins (10). Indeed, the specific rotation of proteins, like native serum albumin ( $\lambda_c$  of 265), at the Cotton effect minimum at 233  $m\mu$ , appeared to be about  $-12,000^\circ$ , whereas that of the ribonuclease ( $\lambda_c$  of 232) was only approximately  $-4500^\circ$  at the same wave length. A simple quantitative relationship, however, is questionable. A preliminary report on discovery of a large positive Cotton effect of  $\alpha$ -helical polyamino acids and proteins at 190-195  $m\mu$  has been published recently by Blout et al (18).

Returning to Fig. 2, it is noteworthy that the rotatory dispersion curves of the helical and nonhelical proteins cross each other in the ultraviolet part. In the visible and near ultraviolet, the helical proteins are less levorotatory than the denatured proteins, whereas in the far ultraviolet at 232-250  $m\mu$  the opposite is true. This comparison, however, is correct only for the native and denatured (disorganized) forms of the highly  $\alpha$ -helical proteins, and one cannot compare in this manner, the native forms of the proteins of Group I with the native forms of the Group III proteins (Table I). For example, the specific rotation of native serum albumin, measured e.g. with light of  $\lambda = 546 m\mu$ , is more negative ( $-78^\circ$ ) than the specific rotation of serum  $\gamma$ -globulin ( $-60^\circ$ ) measured with the same light. Also it is noteworthy that the denaturation of the Group III proteins, such as  $\gamma$ -globulin or pepsin, results in a quite different change of the rotatory constants than in the case of Group I proteins (14, 15, 16).

These effects may differ depending on the protein as well as on the denaturing agent. Especially interesting are the effects of detergents on the optical rotatory properties of various proteins (19, 20, 21). While serum albumin (Group I) is not affected very much by detergents (e.g. sodium dodecyl sulfate),  $\gamma$ -globulin (Group III) is affected strongly. The dispersion constant  $\lambda_c$  increases, and the  $b_0$  becomes more negative, while the specific rotation may shift into either positive or negative direction. Also it is noteworthy that the effect of the detergent depends on the size of the hydrophobic "Tail" of the detergent, a fact which points to the importance of the hydrophobic bonds in the structural stability of these macromolecules. As has been pointed out by Kauzmann (22) and Tanford (17), the inability of the hydrophobic side chains (hydrocarbon residues) of the protein to form bonds with water may be the major cause of forming compact conformations of the long polypeptide chains. As indicated by the author of this paper (21), the hydrophobic bonds within the macromolecules may be especially important for some of the native globular conformation of the Group III (nonhelical) proteins, whereas the structural order in the helical proteins is determined to a large extent by the interchain hydrogen bonds and covalent disulfide (and possibly other) bonds.

#### The Plant Proteins

The optical rotatory properties of plant proteins have been investigated very little. Only incidental data can be found in the older literature, and then chiefly for characterization. The first systematic study attempting to correlate optical rotation with structural changes was started by this author in 1949 at the Victoria University of Manchester, England (23). The potato protein tuberin, and the legumins of pea and vetch were studied by observing



the change of viscosity, reducing capacity (free SH groups), and optical activity on denaturation with various agents, such as guanidine hydrochloride, detergents, acid, and propanol. The optical activity was observed only with the commonly available  $\lambda = 589 \text{ m}\mu$ , i.e. the sodium D-line. It was found that levorotation increased on denaturation, and that optical rotation changes were accompanied by corresponding increases in viscosity and reducing capacity. This work was continued later in 1950-52 at Texas Lutheran College, Seguin, using arachin and amandin (24). In Table II are compiled some of the characteristic data. The pH of the solutions was near neutrality (pH 5.8 - 7.2); the temperature was within the limits of 18 - 24°C, and small temperature changes within these limits did not affect the result significantly. These studies were the first ones which indicated that disorganization of the native structures of proteins by means of denaturing agents results in a negative shift of the specific rotation.

Later on, some plant proteins were included in the general program dealing with the relationships between the optical rotatory dispersion constants and protein conformation. An unfractionated arachin specimen and samples of  $\beta$ -amylase, ficin, papain, and soybean trypsin inhibitor were studied in 1957-58 (7, 8, 14, 15), these being followed by fractionated lima bean trypsin inhibitor (25). While the  $\lambda_c$  values of the arachin and soybean trypsin inhibitor were found to be low, the lima bean protein had high  $\lambda_c$  values. The soybean trypsin inhibitor was studied also with the short wave ultraviolet light, and it was found that it did not exhibit a definite negative Cotton effect.  $\beta$ -amylase of the sweet potato, on the other hand, had a high  $\lambda_c$  and a  $b_0$  of -195, and it yielded a Cotton effect



with a minimum at 233 m $\mu$  (12). Ficin also showed a high  $\lambda_c$  indicating  $\alpha$ -helical conformation, whereas papain in its rotatory properties resembled proteins like ribonuclease. The Cotton effects of ficin and papain have not been studied.

The effect of detergents on the rotatory properties of the soybean trypsin inhibitor, edestin, and  $\alpha$ -conarachin was studied recently, and it was ascertained that all three of these plant proteins belong to the Group III, i.e. that very likely they are nonhelical when in the native state. The Moffitt parameter  $b_0$  of these proteins, when in the native state, was found near zero, and denaturation with sodium decyl or dodecyl sulfates resulted in a negative shift of  $b_0$ . Denaturation of edestin with 8 M urea resulted in no significant change of  $b_0$ , but the levorotation was strongly shifted to the negative direction. The change of the specific rotation of native and urea denatured edestin with wave length is illustrated in Table III. These data, when corrected for the residual mean molecular weight and refractive index, and when plotted according to the method of Moffitt, yield for the native edestin a  $b_0$  of 0, and for the urea denatured edestin a  $b_0$  of -6, which is not a significant difference from zero. The rotatory dispersion constants are compiled in Table IV. Some of the enzymes isolated from yeasts, bacteria, and molds are not included in this Table.

#### Discussion and Conclusions

A survey of the results indicates that the optical rotatory dispersion data imply a considerable variety in the conformation of plant proteins. The diversity seems to be about the same as in animal proteins. While the soybean trypsin inhibitor has little or none of the  $\alpha$ -helical conformation,

the lima bean trypsin inhibitors belong to the group of highly helical proteins. Edestin and  $\alpha$ -conarachin seem to be nonhelical, when in the native state, but it is curious that denaturation with detergents produced a negative shift of the  $b_0$ , and indication of a possible  $\alpha$ -helix formation in some parts of the polypeptide chains after this type of denaturation. Since completely  $\alpha$ -helical polyamino acids and some fibrous proteins have  $b_0$  near  $-650^\circ$ , the values obtained in the treatment with detergents (about  $-120^\circ$ ) indicate only a partial ordering of the chains.

The optical rotatory properties of soybean trypsin inhibitor have been studied recently also by Wu and Scheraga (28). These authors studied, among other aspects, also the conformational transitions of acidified solutions of the inhibitor at higher temperatures. They found for an acid solution of pH 1.22 at  $25^\circ$  a  $b_0$  of  $-70^\circ$ , and this value increased upon heating to  $50^\circ\text{C}$  to  $-106^\circ$ . The levorotation, however, at the same time became somewhat less negative. This change is essentially the same as observed here in a treatment of the inhibitor with sodium decyl sulfate (see Table IV). According to Wu and Scheraga (28), the polypeptide chain of the soybean trypsin inhibitor contains  $\alpha$ -helical twists of both right and lefthanded sense. Upon denaturation, the less stable lefthanded helices are believed to be disorganized first, a transition which should result in a positive shift of the specific rotation and negative shift of the  $b_0$ . As we see, this is borne out by some of the observations. However, there are other facts which contradict this explanation of Wu and Scheraga. Thus, denaturation of the soybean inhibitor with guanidine thiocyanate slightly raises  $\lambda_c$ , which is equivalent to a slight negative shift of  $b_0$ , but the specific rotation is shifted to a negative rather than positive direction (Table IV). Also this explanation

is invalid for the negative shift of  $b_0$  on denaturation of  $\alpha$ -conarachin and edestin with detergents, since the specific rotation at the same time is becoming more negative rather than positive (Table IV). Tanford et al (17) have discussed this possibility of the presence of both right and left-handed helices in the macromolecules of  $\beta$ -lactoglobulin and similar proteins possessing  $b_0$  values near zero, and they concluded that this possibility is not plausible.

Regarding the aspect of analytical characterization of the seed proteins, the globulins of legumes, arachin and  $\alpha$ -conarachin, edestin of hempseed, and amandin of almonds exhibit optical activity of about the same magnitude. The specific rotation of these proteins is shifted into the negative direction upon denaturation with various agents.

The structural order in the macromolecules of the mentioned plant proteins having  $b_0$  near zero is an unsolved problem. Soybean trypsin inhibitor, conarachin, and edestin seem to have little or no  $\alpha$ -helix. How the polypeptide chains in these macromolecules are folded is not known. Two major views must be considered: 1) that these proteins with the  $b_0$  near 0, although compact and tightly folded (low viscous solutions), are disordered; and 2) that some order other than the  $\alpha$ -helical conformation must be present in these proteins. It is impossible to solve this problem by the method of spectropolarimetry alone. The X-ray structural analysis is the only direct method which could give the answer, and it is hoped that it will be applied in the near future at least to such relatively small macromolecules as those in the crystals of soybean trypsin inhibitor. The X-ray structural analysis has shown the presence of the  $\alpha$ -helix in the



segments of the polypeptide chain of myoglobin (1). Also it has shown that the helical chain itself is folded in a rather irregular fashion into a compact body. One can imagine various spatial orders other than helical in the proteins, which, according to optical rotatory dispersion data, are nonhelical. Aside of the possibility of a secondary order other than  $\alpha$ -helix, one can think of various orderly ways of folding the chain (order in the tertiary structure).

The biosynthesis of a polypeptide chain seems to occur in plant cells in the same manner as in animal cells, and the conformation or spatial conditioning of the chain should depend chiefly, when not altogether, on the primary structure, i.e. amino acid composition and sequence. The reasons why in one instance the chains form the  $\alpha$ -helical twists and in some other instances do not, are only partially known. Thus, it is known that proline residues hinder the  $\alpha$ -helix formation. Some answers may be expected upon a more complete elucidation of the biosynthetic mechanisms in the various cells and tissues (29).

#### Summary

The method of spectropolarimetry and general results on the optical rotatory dispersion of proteins in relation to macromolecular conformation have been outlined. Earlier results on the optical rotation of tuberin, legumin, arachin, amandin,  $\beta$ -amylase, ficin, papain, and the trypsin inhibitors from lima bean and soybean are briefly summarized. New results on the optical rotatory dispersion of  $\alpha$ -conarachin and edestin are presented. It was found that  $\alpha$ -conarachin and edestin, when in the native state, have  $b_0$  values near zero, and that the  $b_0$  is shifted to the negative direction upon denaturation with detergents.



## References

1. J. C. Kendrew, R. E. Dickerson, B. E. Strandberg, R. G. Hart, D. R. Davies, D. C. Phillips, and V. C. Shore, *Nature (London)*, 185, 422 (1960).
2. S. Beychok and E. R. Blout, *J. Mol. Biol.*, 3, 769 (1961).
3. P. J. Urnes, K. Imahori, and P. Doty, *Proc. Nat. Acad. Sci. (Washington)*, 47, 1635 (1961).
4. J. A. Schellman and C. G. Schellman, *J. Polymer Sci.*, 49, 129 (1961).
5. P. Urnes and P. Doty, *Adv. Protein Chem.*, 16, 401 (1961).
6. J. T. Yang and P. Doty, *J. Amer. Chem. Soc.*, 79, 761 (1957).
7. B. Jirgensons, *Tetrahedron*, 13, 166 (1961).
8. B. Jirgensons, *Makromol. Chem.*, 44/46, 123 (1961).
9. W. Moffitt and J. T. Yang, *Proc. Nat. Acad. Sci. (Washington)*, 42 596, (1956).
10. N. S. Simmons and E. R. Blout, *Biophys. J.*, 1, 55 (1960); N. S. Simmons, C. Cohen, A. G. Szent-Gyorgyi, D. B. Wetlaufer, and E. R. Blout, *J. Amer. Chem. Soc.*, 83, 4766 (1961).
11. S. B. Zimmerman and J. A. Schellman, *J. Amer. Chem. Soc.*, 84, 2259 (1962).
12. B. Jirgensons, *Arch. Biochem. Biophys.*, 96, 314, 321 (1962); *Biochemistry*, 1, 917 (1962).
13. D. D. Fitts and J. G. Kirkwood, *Proc. Nat. Acad. Sci. (Washington)*, 42, 33 (1956); 43, 1046 (1957); *J. Amer. Chem. Soc.*, 78, 2650 (1956); W. Moffitt, *J. Chem. Phys.*, 25, 467 (1956).
14. B. Jirgensons, *Arch. Biochem. Biophys.*, 74, 57, 70 (1958).
15. B. Jirgensons, *Arch. Biochem. Biophys.* 78, 235 (1958); 85, 532 (1959).
16. B. Jirgensons, *Arch. Biochem. Biophys.* 85, 89 (1959).
17. C. Tanford, P. K. De, and V. G. Taggart, *J. Amer. Chem. Soc.*, 82, 6028 (1960).
18. E. R. Blout, I. Schmier, and N. S. Simmons, *J. Amer. Chem. Soc.*, 84, 3193 (1962).
19. B. Jirgensons, *Arch. Biochem. Biophys.* 94, 59 (1961).
20. B. Jirgensons, *Makromol. Chem.* 51, 137 (1962); *Texas Reports Biol. Med.*, 17, 106 (1959).
21. B. Jirgensons, *Makromol. Chem.*, 56, 138 (1962).
22. W. Kauzmann, in "The Mechanism of Enzyme Action" (W. D. McElroy and B. Glass, eds.), J. Hopkins Univ. Press, Baltimore, p. 70 (1954).
23. B. Jirgensons, *J. Polymer Sci.*, 5, 179 (1950); 6, 477 (1951).
24. B. Jirgensons, *Arch. Biochem. Biophys.*, 39, 261 (1952).
25. B. Jirgensons, T. Ikenaka, and V. Gorguraki, *Makromol. Chem.*, 39, 149 (1960).
26. J. M. Dechary, K. F. Talluto, W. J. Evans, W. B. Carney, and A. M. Altschul, *Nature (London)*, 190, 1125 (1961).
27. W. J. Evans, W. B. Carney, J. M. Dechary, and A. M. Altschul, *Arch. Biochem. Biophys.*, 96, 233 (1962).
28. Y. V. Wu and H. Scheraga, *Biochemistry*, 1, 905 (1962).
29. P. C. Zamecnick, *Biochem. J.*, 85, 257 (1962); M. A. O'Neal and A. C. Griffin, *Cancer Research (in press)*; B. Jirgensons, *Natural Organic Macromolecules*, Pergamon Press, London, 1962, chapt. 12-22.

TABLE I

## Classification of Globular Proteins on the Basis of Rotatory Dispersion Data

Group I, highly  $\alpha$ -helical proteins.  $\lambda_c$  above 240 m $\mu$ ;  $b_0$  above -100

Group II, proteins with a low  $\alpha$ -helix content.  $\lambda_c = 220$ -240;  $b_0$  below -100<sup>c</sup>

Group III, the nonhelical globular proteins.  $\lambda_c =$  below 220 m $\mu$ ;  $b_0$  near 0.

Albumin, egg white

Albumin, serum

Aldolase

$\beta$ -Amylase

Carboxypeptidase

Dehydrogenase, malic

Ficin

Insulin

Lysozyme

Prolactin

Somatotropin

Trypsin inhibitor, lima  
bean

Arachin

Casein,  $\alpha$

Chymotrypsin

Chymotrypsinogen

Deoxyribonuclease

Elastase

Lactoglobulin,  $\beta$

Ovomucoid

Papain

Ribonuclease

Thyroglobulin

Trypsin

Bence-Jones proteins

Casein,  $\beta$

Conarachin,  $\alpha$

Edestin

Globulin,  $\gamma$ , serum

Globulin,  $\gamma$ , milk

Luteinizing hormone

Macroglobulins

Myeloma Globulins

Pepsin

Rennin

Trypsin inhibitor, soy  
bean

TABLE II

Optical rotation and viscosity of native and denatured plant proteins (23, 24)

Protein	Solvent	$\eta_{sp}/c$ , dl/g, $c \rightarrow 0$	$[\alpha]_D$ , degrees
Tuberin, potato	2 M NaCl	0.03	- 43°
do	2.5 M Guanidine-HCl	0.22	80
do	2% Na-dodecylbenzene sulfonate	0.10	50
Legumin, pea	2 M NaCl	0.03	42
do	50% CH <sub>3</sub> COOH	0.43	60
do	1% Na-dodecylbenzene sulfonate	0.10	52
Legumin, vetch	2 M NaCl	0.03	46
do	2.5 M Guanidine-HCl	0.19	89
do	5.0 M Guanidine-HCl	0.35	91
do	2% Na-dodecylbenzene sulfonate	0.10	60
$\eta_{sp}/c$ , dl/g, $c = 1.0\%$			
Arachin, peanuts	1 M NaBr	0.047	41.8
do	2.5 M Guanidine-HCl	0.158	74.2
do	0.5% Na-lauryl sulfate	0.131	61.6
Amandin, almonds	1 M NaBr	0.054	45.1
do	2.5 M Guanidine-HCl	0.149	72.6
do	0.5% Na-lauryl sulfate	0.086	61.0

TABLE III

Optical rotation of native and urea denatured edestin\* at various wave lengths

Native edestin in 0.3 M sodium phosphate, pH 8.0		Edestin in 8 M urea, pH 7.6.
$\lambda$ , m $\mu$	$-\ [\alpha]_{\lambda}$ , degrees	$-\ [\alpha]_{\lambda}$ , degrees
578	52.4	115.4
546	58.8	132.4
492	75.3	170.5
436	105.6	233.8
405	129.2	285.7
390	143.3	314.9
365	178.4	386.7
334	225.8	

---

\* The edestin specimen was isolated from unheated hempseed in the Seed Protein Pioneering Research Laboratory of Southern Utilization Research and Development Division, New Orleans, Louisiana, U. S. Department of Agriculture, and obtained for this study through the courtesy of Dr. Joseph M. Dechary. The specimen was dissolved in 0.3 M sodium phosphate buffer of pH 8.0 and dialyzed against the solvent overnight at room temperature. The concentration of the edestin was calculated from semi-micro Kjeldahl nitrogen analyses.



TABLE IV

Rotatory dispersion constants of various plant proteins

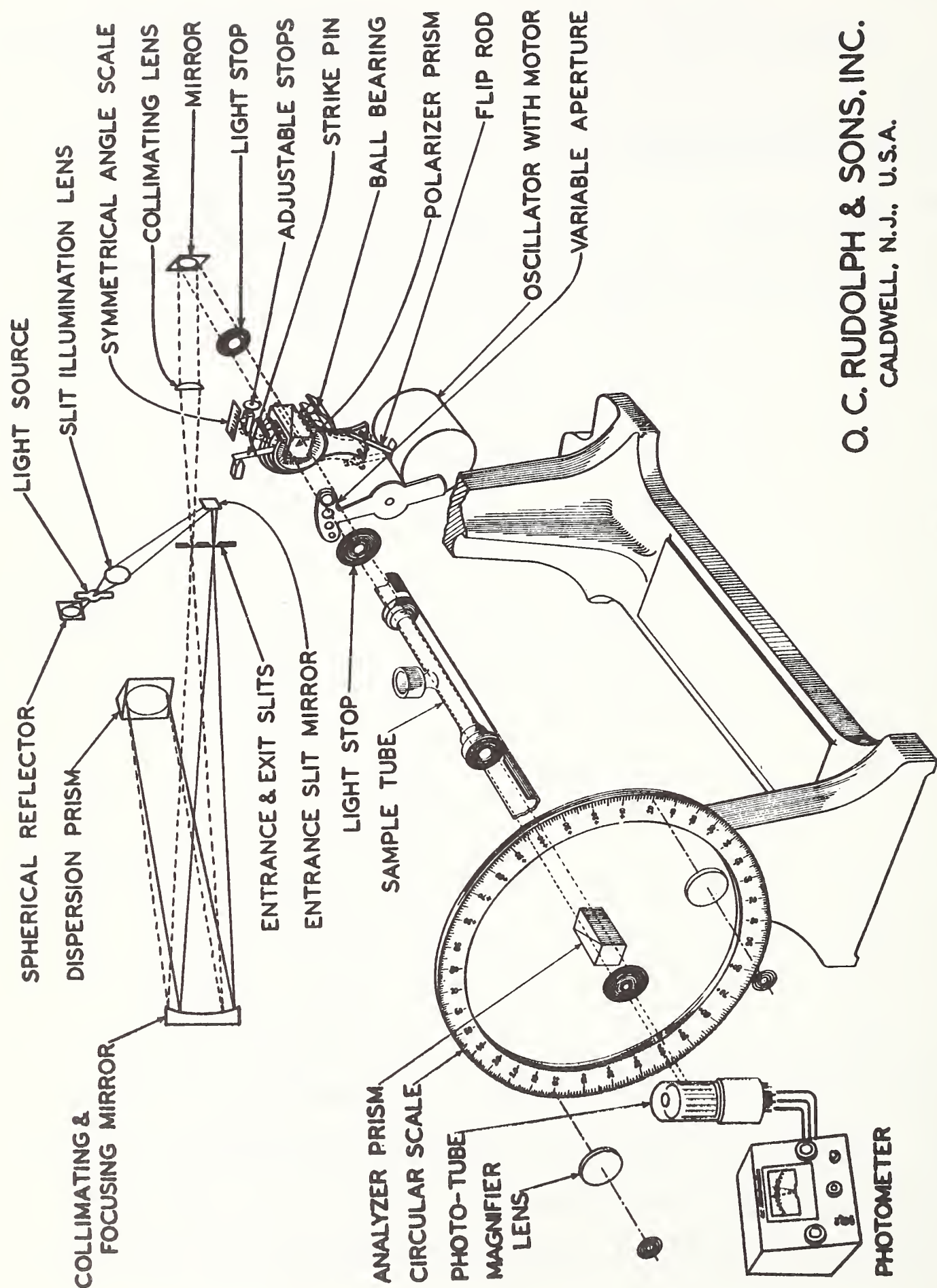
Protein	Solvent	$-\ [\alpha]_{546}$ , degrees	$\lambda_c$ , m $\mu$	$b_0$ , degree
Arachin	Glycine buffer, pH 9.2	55.2	232	
$\alpha$ -Conarachin*	0.1 M Na-phosphate, pH 8.0	42.4		-10
do	0.05 M Na-decyl sulfate	53.4		-123
Edestin	0.3 M Na-phosphate, pH 8.0	58.8		0
do	0.02 M Na-dodecyl sulfate	69.6		-105
do	8 M urea, pH 7.6	132.4		-6
Trypsin inhi- bitor, lima bean, Fr. III	0.05 M Na-phosphate, pH 6.9	32.0	258	
do, Fr. IV	0.05 M Na-phosphate, pH 7.2	32.4	258	
do, Fr. V	0.05 M Na-phosphate, pH 7.5	20.5	280	
do, Fr. VI	0.05 M Na-phosphate, pH 7.6	20.3	274	
Trypsin inhib., soybean	glycine buffer, pH 7.1	96	217	
do	glycine buffer, pH 1.9	105	213	
do	glycine buffer, pH 11.7	97	215	
do	2 M Guanidine-HCNS	105	223	
do	0.1 M NaBr, pH 6.0	95		0
do	0.1 M Na-decyl sulfate	74		-123

Table IV Continued)

Protein	Solvent	$-\ [\alpha]_{546}$ , degrees	$\lambda_c$ , m $\mu$	$b_o$ , degrees
$\beta$ -Amylase, sweet potato	0.1 M Na-phosphate, pH 8.0	32	277	-195
Vicin, fig tree latex	Water, pH 6.7	49	261	
Papain, papaya	0.05 M acetic acid, pH 3.6	75	231	-100

---

The  $\alpha$ -conarachin specimen is described in a paper of Dechary et al (26) and of Evans et al (27). The sample was obtained from the Seed Protein Pioneering Research Laboratory of Southern Utilization Research and Development Division, New Orleans, Louisiana, U. S. Department of Agriculture, through the courtesy of Drs. Altschul and Dechary.



O. C. RUDOLPH & SONS, INC.  
CALDWELL, N.J., U.S.A.

# SERIES 200AS PHOTOELECTRIC SPECTROPOLARIMETER WITH OSCILLATING POLARIZER

FIGURE 1

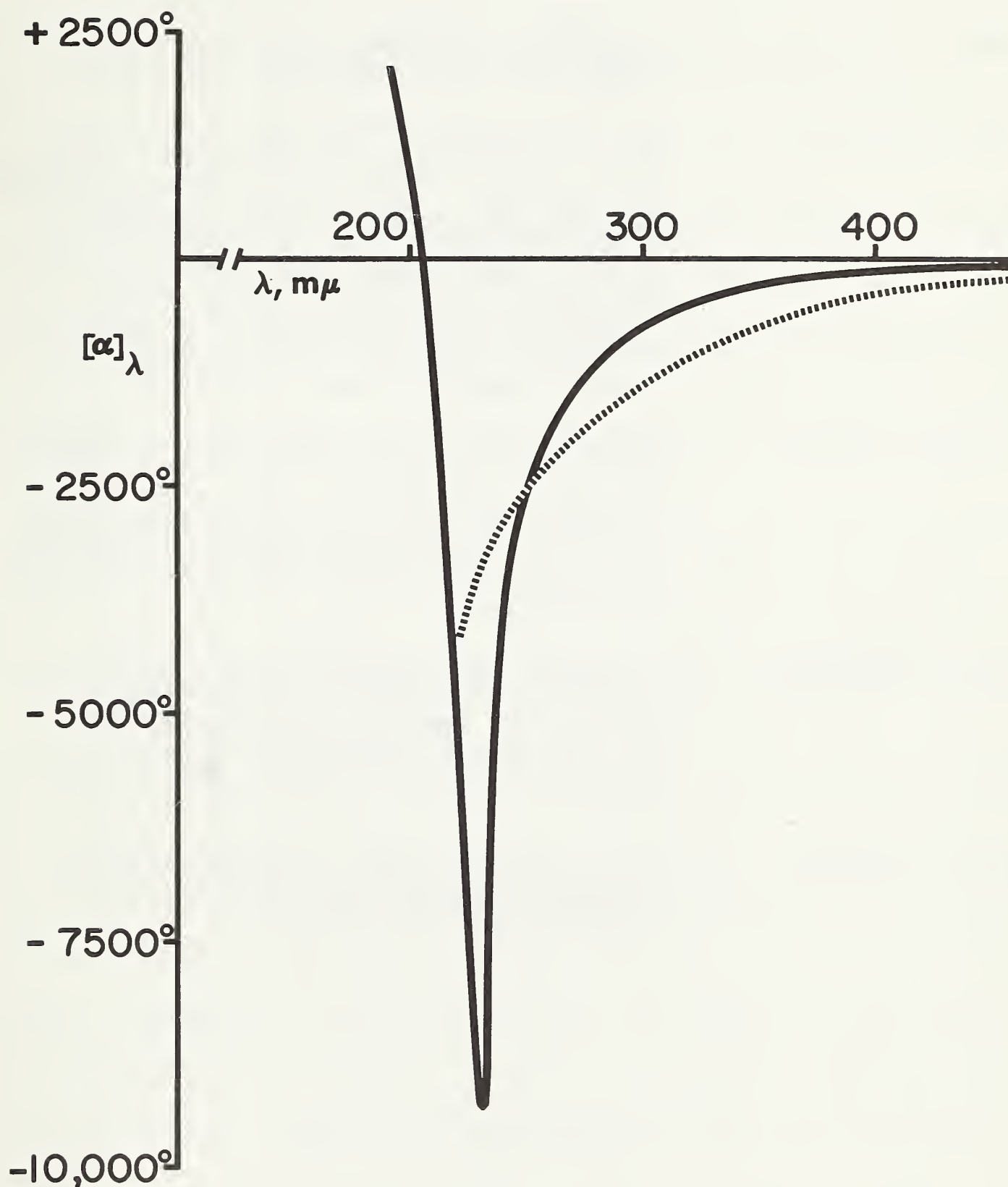


FIGURE 2

Dependence of the specific rotation of proteins on the wave length of light. On the abscissa is plotted the wave length in  $m\mu$ , on the ordinate the specific rotation in angular degrees. The continuous curve represents an idealized case of a native protein with a high  $\alpha$ -helix content in the macromolecules. The dotted curve represents the behavior of the same protein after denaturation. Note that there is no Cotton Effect at the absorption maximum of proteins at 270-280  $m\mu$ .



## Discussion

- Dr. Erhard Gross: Do you have any comment on the difference between lysozyme, chymotrypsin, and trypsin.
- Dr. Bruno Jirgensons: According to the optical rotatory dispersion data, lysozyme has a relatively high content of the  $\alpha$ -helix, whereas chymotrypsin and trypsin have much less of the  $\alpha$ -helical conformation. It is noteworthy that the trypsin inhibitors isolated from the lima beans have much more of the  $\alpha$ -helical structures than the soybean trypsin inhibitor which seems to be largely nonhelical. Most of the plant proteins thus far investigated seem to be nonhelical.
- Dr. Joseph J. Rackis: There is a serious question about the purity of the trypsin inhibitor; the differences found may be due to impurities. Also, going from a disorganized state to a helical state seems improbable in the case of the trypsin inhibitor in Group III since pepsin digests it only after denaturation.
- Dr. Bruno Jirgensons: I requested pure soybean trypsin inhibitor fractions from you, but you were unable to furnish any. However, it is unlikely that the small amount of impurities present in recrystallized inhibitor could distort the result.
- Dr. Mark A. Stahmann: There is evidence that the  $\alpha$ -helix of some polypeptides is more susceptible to pepsin digestion than the random coil.

# ANALYTICAL AND PREPARATIVE ELECTROPHORESIS ON POLYACRYLAMIDE GEL

William J. Evans  
Southern Utilization Research and Development Division  
New Orleans, Louisiana

## ABSTRACT

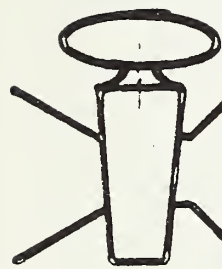
Analytical work is carried out in a commercially available cell made by the E. C. Apparatus Company. Several small slots are formed at the top of the gel by inserting a "former" during the casting operation. Protein solution is layered under the buffer into these slots by a pipet. Dextrose is added to the protein solutions to increase their density, thereby facilitating the layering operation. For the most part analytical electrophoretic work has been performed at 300 volts and from 150 to 200 milliamperes for a period of three hours. A 5% polyacrylamide gel solution with 2.5 ml. each of a 10% solution of each of the catalysts, acrylamide and N, N'-methylenebisacrylamide, is used for gel formation. On completion of electrophoresis the gel is stained with a 1% solution of amido black dye in a solvent composed of water-methyl-alcohol-acetic acid. The excess dye is eluted from the gel by continuous washing with the same solvent mixture. Polyacrylamide gel has proved remarkably efficient in the resolution of highly interacting proteins such as those of the seed proteins (peanut, for example), which had heretofore shown only a few components by free electrophoresis. Analysis by polyacrylamide gel has shown in excess of twenty.

Zone spreading on elution is a serious disadvantage when protein mixtures are fractionated by column electrophoresis. In addition, it has

not been possible thus far to utilize the principles of column electrophoresis with gels because of the great difficulty in eluting the separated fractions by hydrostatic pressure applied to the top of the gel column.

For preparative electrophoretic work using polyacrylamide gel we have modified the LKB vertical column electrophoresis as shown in the figure. Over the projecting end of the right-hand electrode vessel a membrane of ordinary dialysis was tied. This forms a small compartment into which the protein migrates and is then swept out by means of a constant flow pump into a fraction collector. It was found necessary to furnish a continuous supply of fresh buffer for prolonged experiments. This was accomplished by means of reservoir flasks attached to the two electrode compartments. Buffer is continuously removed from beneath the membrane, as this appears to aid in maintaining the constancy of current. Phosphate buffer was found to be unsuitable as using it resulted in the gel being swept up out of the column. Presumably this is due to electroosmotic flow. Tris buffer was found suitable.

By means of this apparatus we have succeeded in fractionating commercial crystalline bovine serum albumin obtaining the monomer in a pure form. Thus it appears that gels can be successfully used in vertical column preparative electrophoresis. Further, this would seem to be a powerful tool, in particular for use with those proteins which are highly interacting, for example, the seed proteins and the structural proteins of mitochondria and ribosomes.



Schematic drawing of electrophoresis apparatus.



## Discussion

Dr. Erhard Gross:

What is the flow rate of the buffer. Is there a permanent change of buffer, or is it recycled?

Dr. William J. Evans:

The flow rate is about 55 ml/hr. The buffer is not recycled.

Dr. Mark A. Stahmann:

Have you tried higher voltages and current, and how is heat dissipated?

Dr. William J. Evans:

At high voltages there was arcing. Current, however, is still the limiting factor.

INFORMAL LUNCHEON PROGRAM

Tuesday, January 22, 1963

12:30 P.M.

Grand Ballroom

Fontainebleau Motor Hotel

"Some Comments on Food Applications of Seed Protein  
Concentrates"

Max Milner, UNICEF, United Nations, New York, N.Y.  
Chairman

M. L. Anson, Consultant, London, England

Z. I. Kertesz, Food and Agriculture Organization,  
United Nations, Rome, Italy

## INTRODUCTION OF SPEAKERS FOLLOWING LUNCHEON

Max Milner

Our friends at the Southern Utilization Research and Development Division are to be congratulated in taking the initiative in holding a conference dealing with the fundamental aspects of seed proteins. It is from the scientific information being presented at this meeting that useful practical applications will grow.

Dr. Altschul suggested that it might be useful in an informal way at this luncheon, to review some of the food applications of seed proteins. This is a subject in which we at UNICEF, cooperating with our colleagues of FAO and WHO, are very much involved, since all seed proteins represent a valuable food resource in many developing countries whose resources may not permit adequate production of proteins of the animal kind to meet the rapidly growing food needs. The presentations and discussions at this conference will doubtless be extremely helpful to this international program by supplying fundamental information which will permit solution of technological problems now facing the effort.

## THE FUTURE OF ISOLATED OILSEED PROTEIN IN FOODS

M. L. Anson

Although Aaron Altschul is a very old friend of mine, he has asked me to predict the future of isolated oilseed protein in foods. This is the second time I have been asked to indulge in such prophecy in public. The first time was for a Symposium on post-war trends in food technology which the I.F.T. rather boldly held in 1944\*.

The predictions of 1944 have turned out to be correct, luckily, for they were based largely on notions and only to a small degree on actual work done. Since 1944, however, a lot of work has been done, which I shall try to summarize briefly.

First, the commercial production of isolated soy protein. The general method of producing the isolated protein has been known for a long time, and, not long after the war, there was a small scale commercial production of good, but expensive, protein for specialty purposes. Now, as a crucial advance, soy protein is produced in millions of pounds per year, at the fairly reasonable price of 35 cents a pound. With an adequate supply of reasonably priced protein assured, large food companies can safely go ahead, and have gone ahead, with research on food products based on isolated protein.

Secondly, if one wants to make products like the most desired animal protein products from oilseed protein, one has to learn how to create desirable structure out of a mushy precipitate of oilseed protein and how to use that structure in foods. A few fundamental discoveries about the

---

\* Proc. Inst. Food Technologists, 5th Conference, Chicago, 1944, page 131



creation and use of desirable structure have been made. In particular, it is now known how to make chewy gel from mushy precipitate and how to make pleasant inhomogenous structures from pieces or fibers of gel. And along with discoveries about how to create texture, there have been discoveries about how to produce desirable flavors cheaply.

Thirdly, and finally, following the basic discoveries which I have mentioned, it has proved possible to make desirable foods from isolated oilseed protein. Some of these will soon face the trials of the marketplace.

Now what predictions can be made about the near future, based this time not on notions but on the actual accomplishments of the post-war years? And what is it impossible to predict?

The production of isolated protein, I feel sure, will go up to a hundred million pounds a year, or to hundreds of millions of pounds. The price will go down, perhaps to 25 cents a pound, in part due to larger production, in part to improved yields, use of protein in the wet state, and the like. The quality will become better, and there will be a greater variety of forms of isolated protein, both variations on the present forms and new forms, such as proteins modified in different ways.

With the research departments of many large food companies working on new foods based on isolated soy protein, it is certain that many such foods will be developed.

Here prophecy must stop, and I must say what I think is unpredictable.

One cannot know whether or not any further discoveries will be made in the near future of new fundamental ways of getting desirable characteristics out of isolated oilseed protein.

One cannot predict with any exactness at all the number and character of the food products that will come out of the current research of many food companies, how long it will take to develop products acceptable to the consumer or what the degree of the commercial success in the near future will be. Conventional market research, at the present stage of the game, cannot be of much help in predicting a commercial future which will depend so much on the unpredictable results of research. Indeed, conventional market research may be a source of confusion, since its usual bases are lacking.

To summarize, I feel sure we are at the take-off stage not of the creation of a few new grocery items but of the creation of a new, revolutionary and major sector of the food industry. Man instead of feeding plant materials into an animal and using the animal as a source of protein foods will, in some measure, feed plant materials into a factory and come out with foods that fulfill the functions of animal protein foods, without necessarily being exact imitations. Man is now in a technical position to replace processing in the animal with processing of human design.

How much of the vast 35 billion dollars a year American animal protein food market will be taken over by plant protein foods, how fast the new development will take place, what variety of forms of plant protein foods will come out of research, all that is beyond reasonable prediction. The replacement of animal protein products will surely be modest in the near future. But even a modest replacement can amount to a great deal.

A conference like this could not have taken place fifteen years ago, there would not have been enough to talk about. And if there had been a

conference with adequate material for discussion, practically nobody from industry would have attended. Now there has been a full, if not overfull, conference. Despite the still slim relation between the academic work and its practical applications, the elite of industry has come to the conference in large numbers. The very existence of this conference and the character of its membership are a more eloquent and reliable prophecy of the future of isolated oilseed protein than any prophecy that can be concocted by me.

UNITED STATES DEPARTMENT OF AGRICULTURE  
AGRICULTURAL RESEARCH SERVICE  
SOUTHERN UTILIZATION RESEARCH AND DEVELOPMENT DIVISION

SEED PROTEIN CONFERENCE

January 21-23, 1963

ATTENDANCE LIST

Abbott, Donald C., Dept. of Biochemistry, Oklahoma State University,  
Stillwater, Oklahoma

Aines, Philip D., The Procter & Gamble Co., Food Division, Box 201,  
Cincinnati 24, Ohio

Anson, M. L., 100 Eaton Square, London, S. W. 1, England

Antonini, Eraldo, Istituto de Chimica Biologica, Universita di Roma,  
Rome, Italy

Arquembourg, Pierre C., Medical School, Tulane University, New Orleans,  
Louisiana

Arquembourg, Rene, Microbiology Dept., Tulane University, New Orleans,  
Louisiana

Balintfy, Joseph L., Asst. Professor, Tulane University's School of  
Business, New Orleans, Louisiana

Barrentine, Ben F., Dept. of Chemistry, Agricultural Experiment Station,  
Mississippi State University, State College, Mississippi

Belavady, Bhavani, Dept. of Medicine, Tulane University, New Orleans,  
Louisiana

Berenson, Gerald S., Medical School, Louisiana State University,  
New Orleans, Louisiana

Bressani, Ricardo, Div. of Agric. & Food Chem., Instituto de Nutricion  
de Centro America y Panama, Carretera Roosevelt, Zona 11, Guatemala,  
Guatemala, C. A.

Brew, William, Manager, Chem. Res. & Control Labs., Ralston Purina Co.,  
St. Louis, Missouri

Brown, W. D., Dept. of Nutritional Sciences, University of California,  
Berkeley, California



Buchanan, Ben F., Manager, Technical Applications, Research Center,  
General Foods Corporation, White Plains, New York

Buck, R. E., Res. & Quality Control Div., H. J. Heinz Company,  
1062 Progress, Pittsburgh 30, Pennsylvania

Burt, L. H., Cellulose & Protein Products Dept., Hercules Powder Co., Inc.,  
910 Market Street, Wilmington 99, Delaware

Calvert, C. A., Ralston Purina Co., Checkerboard Square, St. Louis 2,  
Missouri

Cantor, S. M., Vice President - Research, DCA Food Industries, Inc.,  
45 West 36th Street, New York 18, New York

Caridad, Carlos, The Nestle Co., Inc., 100 Bloomingdale Road, White  
Plains, New York

Cherry, Joe H., Horticulture Dept., Purdue University, Lafayette, Indiana

Ching, Te May, Associate Professor, Dept. of Farm Crops, Agricultural  
Experiment Sta., Oregon State University, Corvallis, Oregon

Circle, Sidney J., Associate Director, Protein Res., Central Soya,  
1825 N. Laramie Ave., Chicago 39, Illinois

Corey, Harold, Thomas J. Lipton, Inc., 1500 Hudson Street, Hoboken,  
New Jersey

Dessauer, Herbert C., Assoc. Prof. of Biochemistry, Louisiana State  
University, School of Medicine, New Orleans, Louisiana

DeWitt, C. W., Assoc. Prof., School of Medicine, Tulane University,  
New Orleans, Louisiana

Diboll, Alfred G., Research Assistant, University of Texas, Plant  
Research Institute, Austin, Texas

Dore, J. B., Manager, Oilseed Spec. Prod. Lab., Ralston Purina Company,  
St. Louis, Missouri

Duvick, Donald N., Pioneer Hi-Bred Corn Co., Johnston, Iowa

Eastin, Jerry D., Agronomist, University of Nebraska, Lincoln, Nebraska

Evans, Robert J., Dept. of Biochemistry, Michigan State University,  
East Lansing, Michigan

Eversole, R. A., Biochemistry Dept., Cargill, Inc., Cargill Building,  
Minneapolis 2, Minnesota

Fincher, H. D., Process Engr., Anderson Clayton & Co., P. O. Box 2538,  
Houston, Texas

Fishkin, A. F., Asst. Prof., School of Medicine, Louisiana State  
University, 1542 Tulane Ave., New Orleans, Louisiana

Fox, Kenneth, U. S. Department of Agriculture, Washington 25, D. C.

Gonzalez del Cueto, Avelina, Instituto Mexicano de Investigaciones  
Tecnologicas A.C., San Francisco 715, Mexico 12, D.F., Mexico

Grabar, Pierre, Head, Service de Chimie Microbienne, Institut Pasteur,  
Paris, France

Gray, J. S., Lance, Inc., P. O. Box 2389, Charlotte 1, North Carolina

Gross, Erhard, Res. Chemist, Section on Metabolites, Lab. of Chemistry,  
National Institutes of Health, Bethesda 14, Maryland

Gulati, C. K., Prof. of Agricultural Chemistry, Post Graduate School,  
Indian Agricultural Research Institute, New Delhi, India

Hamilton, James G., Tulane University's School of Medicine, 1430 Tulane  
Avenue, New Orleans, Louisiana

Harris, George C., Hercules Powder Co., Delaware Trust Building,  
Wilmington 99, Delaware

Hartley, W., Scientific Attache', Australian Embassy, Room 205,  
1907 K Street, N. W., Washington 6, D. C.

Hawrylewicz, Erwin J., Armour Research Foundation of Illinois Institute  
of Technology, 10 West 35th Street, Chicago 16, Illinois

Hebo, Halfdan, Vice President, American Protein Corp., 30 Rockefeller  
Plaza, New York 20, New York

Holley, K. T., Georgia Experiment Station, Experiment, Georgia

Hopper, J. H., Dir. of Nutrition, Kellogg Co., Battle Creek, Michigan

Horan, F. E., Res. Manager, Agric. Products Res. Section, Archer Daniels  
Midland Co., 733 Marquette Ave., Minneapolis 40, Minnesota

Hornung, M., Microbiology Dept., Tulane University, 1430 Tulane Avenue,  
New Orleans, Louisiana

Howell, Robert W., U. S. Regional Soybean Lab., Crops Research Division,  
U. S. Dept. of Agriculture, Urbana, Illinois



Jiminez, Jesus M., Head, Biochemistry Dept., University of Costa Rica,  
School of Medicine, 2321 St. Charles Ave., New Orleans, Louisiana

Jirgensons, Bruno, Biochemist, M. D. Anderson Tumor Institute, Texas  
Medical Center, Houston 25, Texas

Jung, Rodney C., Head, Div. Tropical Medicine, Tulane Medical School,  
1430 Tulane Avenue, New Orleans, Louisiana

Kelley, John, Central Research Laboratories, General Mills, Inc.,  
9000 Plymouth Ave., Minneapolis 27, Minnesota

Kertesz, Z. I., Food Sc. & Tech. Br., Nutrition Div., Food & Agric. Org.  
of the United Nations, Via delle Terme di Caracalla, Rome, Italy

Kingdon, Fred, Research Chemist, Hercules Powder Co., Delaware Trust  
Building, Wilmington 99, Delaware

Konaka, Kazuichi, Univ. of Alabama Medical Center, 1919 - 7th Avenue S.,  
Birmingham 3, Alabama

Kovacs, Louis E., Pres. & Gen. Mgr., Vitamins, Inc., 809 West 58th St.,  
Chicago 21, Illinois

Krulick, Samuel, Plant Prods. Lab., Eastern Util. Res. & Dev. Div.,  
600 East Mermaid Lane, Philadelphia 18, Pennsylvania

Liener, Irvin E., Dept. of Agricultural Chemistry, University of  
Minnesota, St. Paul, Minnesota

McWard, G. Wayne, Asst. Professor, University of Maine, Orono, Maine

Maddy, Kenneth H., Feed Chemicals Dev., Agric. Chemicals Division,  
Monsanto Chemical Co., 800 N. Lindbergh Blvd., St. Louis 66, Missouri

Marcus, Abraham, Biochemist, Plant Industry Station, Agric. Marketing  
Serv., U. S. Dept. of Agriculture, Beltsville, Maryland

Melnychyn, Paul, Research Laboratories, Carnation Company,  
8015 Van Nuys Blvd., Van Nuys, California

Meyer, Edwin W., Central Soya, 1825 N. Laramie Ave., Chicago 39, Illinois

Milner, Max, Food Cons. Div., UNICEF, United Nations, New York

Mollenhauer, Hilton H., Electron Microscope Lab., Biology Building -  
Room 22, University of Texas, Austin, Texas

Muldrey, James E., Assoc. Prof. of Biochemistry & Med., Dept. of Medicine,  
Tulane Medical School, 1430 Tulane Ave., New Orleans, Louisiana

Mullen, Joseph D., Central Research Lab., General Mills, Inc.,  
9000 Plymouth Avenue N., Minneapolis 27, Minnesota

Nimmo, Charles C., Cereals Lab., Western Utilization Research and  
Development Div., Agricultural Research Serv., U. S. Dept. of Agric.,  
Albany, California

O'Leary, Daniel K., Asst. Professor of Medicine, University of Alabama  
Medical Center, 1919 - 7th Avenue S., Birmingham 3, Alabama

Olson, Alfred C., WURDD, USDA, Div. of Biology, California Institute of  
Technology, Pasadena, California

Perrone, J. C., Instituto Nacional de Tecnologia, Rio de Janeiro, Brazil

Phelps, Richard A., Asst. Dir. Research & Ed., National Cottonseed  
Products Assn., 1104 Praetorian Bldg., Dallas, Texas

Rabson, Robert, Assoc. Prof., Dept. of Biology, University of Houston,  
Houston 4, Texas

Rackis, J. J., Oilseed Crops Lab., Northern Utilization Research and  
Development Div., ARS, USDA, 1815 N. University St., Peoria 5, Illinois

Radhakrishnamurthy, B., L. S. U. School of Medicine, Tulane Avenue,  
New Orleans, Louisiana

Raspet, A., Graduate Scientist, Seed Technology, Mississippi State  
University, State College, Mississippi

Roberts, Ronald C., University of Minnesota, 116 N. Lexington Pkwy.,  
St. Paul, Minnesota

Sellinger, O. Z., Asst. Prof. Biochemistry, Tulane University,  
1430 Tulane Avenue, New Orleans, Louisiana

Sharon, Nathan, The Weizmann Institute of Science, Rehovoth, Israel

Shendy, K. G., Tulane University Medical School, New Orleans, Louisiana

Siegelman, H. W., Crops Research Div., Agric. Res. Serv., Plant Industry  
Station, Beltsville, Maryland

Smith, R. H., Biochemical Dept., British Glues & Chemicals Ltd.,  
123, Grange Rd., London, S. E. 1, England

Spies, Joseph R., Allergens Lab., EURDD, U.S.D.A. South Building,  
14th Street & Independence Ave. S.W., Washington 25, D. C.

Stahmann, Mark A., Dept. of Biochemistry, College of Agriculture,  
University of Wisconsin, Madison 6, Wisconsin



Stark, George R., Asst. Professor, The Rockefeller Institute,  
New York 21, New York

Stelzner, Harry D., Georgia Experiment Station, Experiment, Georgia

Strobel, R., Procter & Gamble, 6721 Rutherford, Cincinnati 39, Ohio

Tombs, Michael, Unilever Research Laboratory, Colworth House, Sharnbrook,  
Bedford, England

Tristram, G. R., Dept. of Biochemistry, Bute Medical Buildings,  
St. Andrews, Fife, Scotland

Vandevoord, Jackques, Biochemist, Touro Research Institute, New Orleans,  
Louisiana

Varner, J. E., Senior Biochemist, Research Institute for Advanced Study,  
7212 Bellona Ave., Baltimore 12, Maryland

Veith, Dianne C., Tulane University, New Orleans, Louisiana

Voit, J. F., Sales Manager, Ralston Purina Co., St. Louis 8, Missouri

Weswig, Paul H., Dept. of Agricultural Chemistry, Oregon State University,  
Corvallis, Oregon

Wiegmann, John R., General Mills, Inc., P. O. Box 191, Kankakee, Illinois

Wilcke, Harold L., Asst. Dir. Res., Ralston Purina Co., St. Louis,  
Missouri

Wilding, M. D., Swift & Company, Exchange & Packers Ave., Chicago 9,  
Illinois

Williams, Virginia R., Dept. of Biochemistry, Coates Laboratories,  
Louisiana State University, Baton Rouge, Louisiana

Wilson, A. L., Asst. Dir. Res., Research Dept., Corn Products Co.,  
P. O. Box 345, Argo, Illinois

Wilson, Curtis M., Crops Research Division, ARS, USDA, 112 Davenport  
Hall, University of Illinois, Urbana, Illinois

Wolf, W. J., Northern Utilization Research & Development Division,  
1815 N. University Street, Peoria 5, Illinois

Woychik, J. H., Cereals Properties Lab., Northern Utilization Research &  
Development Division, 1815 N. University St., Peoria 5, Illinois

Yare, Robert S., Research Center, General Foods Corp., White Plains,  
New York

Zacharius, Robert M., Plant Prods. Lab., Eastern Utilization Research & Development Division, 600 E. Mermaid Lane, Philadelphia 18, Pennsylvania

SU ATTENDANCE

Dr. A. M. Altschul, Seed Protein Laboratory

Mr. J. J. Berquist, Photo Laboratory

Mr. N. R. Bertoniere, Food Crops Laboratory

Mrs. Margaret M. Blair, Seed Protein Laboratory

Mr. W. B. Carney, Seed Protein Laboratory

Miss Fairie Lyn Carter, Oilseed Crops Laboratory

Mr. E. A. Catalano, Food Crops Laboratory

Mr. G. P. Clasen, Assistant to Director, Management

Mrs. Mary Louise Connor, Seed Protein Laboratory

Dr. J. M. Dechary, Seed Protein Laboratory

Dr. H. J. Deobald, Food Crops Laboratory

Mr. F. G. Dollear, Oilseed Crops Laboratory

Dr. H. P. Dupuy, Oilseed Crops Laboratory

Dr. Ahmed S. El-Nockrashy, Oilseed Crops Laboratory

Dr. W. J. Evans, Seed Protein Laboratory

Mr. A. F. Fayette, Photo Laboratory

Dr. C. H. Fisher, Director

Mr. R. O. Feuge, Oilseed Crops Laboratory

Dr. V. L. Frampton, Oilseed Crops Laboratory

Mr. E. A. Gastrock, Engineering and Development Laboratory

Dr. G. E. Goheen, Assistant Director, Program Operations

Dr. D. Hamm, Seed Protein Laboratory

Dr. J. F. Harrington, Seed Protein Laboratory

Miss Vera C. Hasling, Food Crops Laboratory

Mrs. Mary C. Hausser, Office of Assistant Director, Program Appraisal

Mr. C. L. Hoffpauir, Assistant Director, Program Development

Mr. J. T. Hogan, Food Crops Laboratory

Miss Marie A. Jones, Information Office

Mrs. Mary Alice Jones, Office of Assistant Director, Industrial Dev.

Mr. W. H. King, Oilseed Crops Laboratory

Mrs. Madeline G. Lambou, Engineering and Development Laboratory

Dr. W. Landmann, Oilseed Crops Laboratory

Mrs. Louise S. Lee, Oilseed Crops Laboratory

Dr. G. E. Mann, Oilseed Crops Laboratory  
Mrs. Wilda H. Martinez, Oilseed Crops Laboratory  
Mr. L. W. Mazzeno, Jr., Office of Assistant Director, Industrial Dev.  
Mr. T. A. McLemore, Food Crops Laboratory  
Miss Nelle J. Morris, Oilseed Crops Laboratory

Mr. N. J. Neucere, Seed Protein Laboratory  
Mr. F. L. Normand, Food Crops Laboratory

Dr. R. L. Ory, Seed Protein Laboratory

Mr. E. L. Patton, Assistant Director, Industrial Development  
Mrs. Marilyn S. Penberthy, Office of Assistant Director, Industrial Dev.  
Mr. R. M. Persell, Assistant Director, Program Appraisal  
Mr. I. G. Peters, Oilseed Crops Laboratory  
Mr. J. Pominski, Engineering and Development Laboratory

Miss Mary L. Rollins, Cotton Physical Properties Laboratory

Mrs. Shirley T. Saucier, Office of Assistant Director, Industrial Dev.  
Mrs. Bea A. Sharar, Office of Assistant Director, Industrial Dev.  
Mrs. Margaret B. Soniat, Office of Assistant Director, Industrial Dev.  
Mr. J. J. Spadaro, Engineering and Development Laboratory  
Mr. A. J. St. Angelo, Seed Protein Laboratory  
Mr. D. M. Stockwell, Seed Protein Laboratory

Mrs. Katherine F. Talluto, Seed Protein Laboratory  
Miss Dorothea J. Teunisson, Oilseed Crops Laboratory

Mr. H. L. E. Vix, Engineering and Development Laboratory

Mrs. Nancy Williams, Economic Research Service

Dr. L. Y. Yatsu, Seed Protein Laboratory

These proceedings were compiled and edited

by

Aaron M. Altschul, Katherine F. Talluto

and Beatrice A. Sharar



